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(57) Abstract

The invention provides a composition of matter, comprised of certain oligonucleotides, which inhibit the expression of β/A4 peptide of Alzheimer's disease and Down's Syndrome, and nerve growth factor (NGF) to reverse morphological changes caused in neuronal cells by β/A4 peptide. Further, pharmaceutical compositions, kits and methods for treatment of β/A4 amyloid-induced morphology as well as an assay for screening candidate antisense oligonucleotides effective in treatment of deleterius effects that are visited upon cells by β/A4 amyloid peptide are described.

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Using Antisense Oligonucleotides to Modulate Nerve Growth and to Reverse β/A4 Amyloid-Induced Morphology

Background of the Invention

Field of the Invention

The invention relates to the treatment of Alzheimer's Disease and Down's syndrome. More particularly, the invention relates to the development of therapeutic agents that are capable of reversing deleterious effects caused by A4 amyloid peptide that are associated with Alzheimer's Disease and Down's syndrome.

Summary of the Related Art

Alzheimer's Disease (AD) presents a public health concern of ever increasing importance. Katzman, *N. Engl. J. Med. 314*:964-973 (1986) teaches that the syndrome is characterized by intellectual deterioration in an adult that is severe enough to interfere with occupational or social performance. Hay and Ernst, *Am. J. Pub. Health 77*:1169-1175 (1987) teach that AD is the most common form of adult-onset dementia and the fourth leading cause of death in the United States.

The dementia associated with AD correlates with neuropathological changes that are found in the brain. Hyman *et al.*, *Science 225*:1168-1170 (1984) disclose that the major input and output pathways of the hippocampus have strikingly high concentrations of senile plaques (SPs) and neurofibrillary tangles (NFTs), which may functionally isolate the hippocampus, thereby impairing memory. Mountjoy *et al.*, *Aging 4*:1-11 (1983) disclose substantial

loss of neurons in AD brain. Coyle *et al.*, *Science 219*:1184-1190 (1983) teach that AD brain shows a decrease in acetylcholine positive sites.

One characteristic commonly associated with AD is the presence of amyloid-containing senile plaques (SPs) in the brain. Majocha *et al.*, *Proc. Natl. Acad. Sci. USA 85*:6182-6186 (1988) teach that these plaques range from about 9 to 50 μm in diameter and vary in morphology and density. Wisniewski and Terry, Progress in Neuropathology (Zimmerman, ed.), Grune and Stratton, New York, N.Y., pp.1-26 (1973) teach that SPs are composed of extracellular amyloid, reactive cells, degenerating neurites that contain NFTs, lysosomes, abnormal mitochondria and astrocytic processes. Mertz *et al.*, *Acta Neuropathol. 60*:113-124 (1983) disclose that the core of SPs is formed by amyloid that is composed of fibrils of 4-8 nm in diameter.

The nature of the amyloid from SPs has been determined. Glenner and Wong, *Biochem. Biophys. Res. Commun. 120*:885-890 (1984) disclose a 4.2 kilodalton AD brain-derived peptide having a unique sequence of 28 amino acids. A polypeptide of similar sequence was also isolated by Glenner and Wong, *Biochem. Biophys. Res. Commun. 122*:1131-1135 (1984), from the cerebrovascular amyloid of a Down's syndrome brain. A single amino acid substitution, of glutamic acid for glutamine at position 11, distinguished the two proteins. Masters *et al.*, *Proc. Natl. Acad. Sci. USA 82*:4245-4249 (1985) disclose an amyloid plaque core-derived peptide of 4.2 kd having a nearly identical sequence as the AD-derived peptide of Glenner and Wong, which was relatively insoluble in a variety of solvents. Castano *et al.*, *Biochem. Biophys. Res. Commun. 141*:782-789 (1986) teach that short synthetic peptides having structures homologous to the 4.2 kd AD-peptides exhibit similar aggregation properties. Kang *et al.*, *Nature 325*:733-736 (1987) teach that the peptide that contributes to senile plaques is a 42 or 43 amino acid stretch. This peptide is cleaved from a larger protein, now known as amyloid precursor protein or APP, a ubiquitous membrane-spanning glycoprotein defined by a locus on chromosome 21 (Goldgaber *et al.*, *Science 235*:877-880 (1987); Kang *et al.*, *Nature 325*:733-736 (1987); Kitaguchi *et al.*,

Nature 331:531-534 (1988); Tanzi *et al., Science 235*:880-882 (1987); Tanzi *et al., Nature 331*:528-530 (1988); Ponte, *et al., Nature 331*:525-527 (1988); Zain *et al., Proc. Natl. Acad. Sci. USA 85*:929-933 (1988)). The peptide will be referred to herein as ß/A4 peptide.

Molecular cloning experiments (Goldgaber *et al., Science 235*:877-880 (1987); Kang *et al., Nature (London) 326*:733-736 (1987); Robakis *et al., PNAS USA 84*:4190-4194 (1987); Tanzi *et al., Science 235*:880-884 (1987); Kitaguchi *et al., Nature 331*:530-532 (1988); Ponte *et al., Nature 331*:525-527 (1988); Tanzi *et al., Nature 331*:527-530 (1988)) indicate that the amyloid β-protein is part of at least three distinct precursors (βAPP_{695}, βAPP_{751}, βAPP_{770}) which are encoded by a gene on human chromosome 21 (Robakis *et al., Lancet 1*:384-385 (1987)). Both βAPP_{751} and βAPP_{770} contain a 56-amino acid insert with high sequence homology to the Kunitz-type serine protease inhibitors (KPI) which is missing from βAPP_{695} (Kitaguchi *et al., Nature 331*:530-532 (1988); Ponte *et al., Nature 331*:525-527 (1988); Tanzi *et al., Nature 331*:527-530 (1988)). βAPP is expressed in almost every tissue examined (Tanzi *et al., Science 235*:880-884 (1987)) and in the brain it is found in both neuronal and non-neuronal cells (Robakis *et al.* (1988) *In: "Disorders of the Developing Nervous System: Changing views on their origins, diagnosis and treatments."* (Swan, J.W. ed) Alan R. Liss, Inc. New York, NY).

It is the carboxy terminal of APP which contains the amyloidogenic beta peptide domain of approximately 42 amino acids (Kang *et al., Nature 325*:733-736 (1987)). This region of the APP can be cleaved within the beta peptide via a constitutive secretory pathway (Esch *et al., Science 248*:1122-1124 (1990)), a process that prevents amyloid formation since the entire beta amyloid peptide is not preserved. More recently it was shown that the 4 kDa beta amyloid peptide is a normal secretory product, produced via a Golgi-endosome mediated process, that is released from cells (Shoji *et al., Science 258*:126-129 (1992); Golde *et al., Science 225*:728-730 (1992); Seubert *et al., Nature 361*:260-263 (1993)).

Recently, there has been considerable interest in using the tools of molecular biology in determining what role ß/A4 peptide might play in the pathology of Alzheimer's disease. Maestre *et al.*, *Brain Res. 599*:64-72 (1992) teach that transfected PC12 cells that produce ß/A4 peptide are larger than untransfected controls, have longer neurites, and have substantially larger numbers of membrane-limited surface extensions that resemble blebs and microvilli. ß/A4 peptide was found to be localized in these membranous processes. Majocha *et al.*, *Mol. Chem. Neuropathol. 18*:99-113 (1993) teach that transfected PC12 cells that express ß/A4 peptide secrete factors that stimulate neurite lengthening and cell differentiation to a neuronal phenotype. Tate *et al.*, *Proc. Natl. Acad. Sci. USA 89*:7090-7094 (1992) teach that transfected cells that express ß/A4 peptide can significantly alter circadian activity when grafted into the brain of rats.

Individuals afflicted with Down's syndrome (DS) have brain pathology that is virtually identical to the changes seen in AD. Both NFTs and SPs that are characteristic of AD are seen in DS cases over the age of 40 years. The NFTs and SPs appear to be morphologically and immunohistochemically indistinguishable between the two disorders. Neurochemical alterations are also analogous. *See*, Ikeda, S. *et al.*, *Lab. Invest. 61*:133-137 (1989); Cole *et al.*, *Acta Neuropathol 85*:542-552 (1993); Patterson *et al.*, *Proc. Natl. Acad. Sci. 85*:8266-8270 (1988); Ikeda *et al.*, *Prog. Clin. Biol. Res. 317*:313-323 (1989); and Beyreuther *et al.*, *Prog. Clin. Biol. Res. 379*:159-182 (1992).

Although APP is widely distributed in eukaryotic cells the normal function of the protein is not yet fully elucidated. Several lines of evidence have implicated a relationship between APP and nerve growth factor (NGF). Treatment with NGF induced the release of 125 and 120 kDa APP species which contained the KPI domain and lacked the carboxy-terminal portion of the protein (Refolo *et al.*, *Biochem. Biophys. Res. Commun. 164*:664-670 (1989)). Administration of NGF to rat brain increased the level of APP mRNA (Refolo *et al.*, *Biochem. Biophys. Res. Commun. 164*:664-670 (1989)). Application of NGF to developing basal forebrain coincided with increased

levels of prion protein (PrP) and APP mRNAs (Mobley *et al., Proc. Natl. Acad. Sci. 85*:9811-9815 (1988)). The level of the 695 isoform was selectively increased, rather than the higher molecular weight APP species. After treatment of PC12 cells with NGF, redistribution of APP occurred since it was localized to growth cones and processes in addition to cytoplasm (Fukuyama *et al., Molec. Brain Res. 17*:17-22 (1993)). Antibodies to APP specifically diminished the effects of NGF on neurite length and branching (Milward *et al., Neuron 9*:129-137 (1992)).

Furthermore, animal research has demonstrated that the ascending cholinergic projections in the brain express low- and high-affinity receptors for NGF and are NGF-sensitive as well as probably NGF-dependent. Cholinergic lesions lead to cognitive disturbances, and treatment with NGF can improve cognitive behavior in animals. Moreover, in a human clinical trial, it was observed that NGF treatment in an Alzheimer's patient gave positive results indicating partial reversal of the effects of AD in the patient (Lars Olson, *Experimental Neurology 124*:5-15 (1993)).

These important discoveries indicate an active role for ß/A4 peptide in AD and DS neuropathology. However, there is a need to determine whether the pathology attributable to ß/A4 peptide is reversible. Development of therapeutic compounds, rather than merely prophylactic agents may well depend upon the reversibility of ß/A4 peptide-induced pathology, particularly cell size expansion, neurite extension and differentiation of cells into a neuronal phenotype. Further, there is a need for an *ex vivo* assay to measure effect of such potentially therapeutic compounds on Alzheimer amyloid production before they can be used in clinical trials.

Summary of the Invention

The invention relates to the discovery that it is possible to halt and reverse the accumulation of the beta/A4 peptide in cells which overexpress the

peptide. Thus, the invention provides a method for reversing the morphological changes brought upon a cell by ß/A4 peptide. In a first aspect, the method according to the invention comprises administering to a cell that is morphologically altered by ß/A4 peptide an oligonucleotide that reduces or eliminates synthesis of the ß/A4 peptide. Modified oligonucleotides that are useful in the method according to the invention are primarily those that are more resistant to nucleolytic degradation than conventional oligonucleotide phosphodiesters. These include oligonucleotides having a variety of modified internucleoside linkages, mixed backbones, nuclease resistant 3′ cap structures, integrated triplex-forming structures or self-stabilized structures, or any combination of these. Preferably, the oligonucleotides have a nucleotide sequence that is complementary to the nucleotide sequence encoding ß/A4 peptide or its precursor protein APP (*see*, Figure 13), or to the initiation codon from which ß/A4 peptide or APP is translated. Surprisingly, reduction of ß/A4 peptide expression is sufficient to reverse the morphological changes induced by ß/A4 peptide, without any apparent need to eliminate other effects that might have been initiated by ß/A4 peptide, but subsequently maintained by other molecules. Accordingly, it is of interest to examine the effect of reducing ß/A4 peptide levels in a mammal, including a human, to determine the effects of reduced ß/A4 peptide upon the entire organism. The invention, in a second aspect, provides a method for making such a determination. Initially, these studies will preferably be conducted in a non-human mammal. Such studies involve administering to the animal such modified oligonucleotides as were previously described for reversing morphological changes brought upon cells by ß/A4 peptide.

In a third aspect, the invention also relates to methods for treating or preventing Alzheimer's disease in an animal, in particular, a human.

In a fourth aspect, the invention also relates to methods for treating Down's syndrome in an animal, in particular, a human.

In a fifth aspect, the invention provides oligonucleotides that are effective in reducing ß/A4 peptide levels in a cell or an animal, and in

reversing morphological changes in a cell that were caused by ß/A4 peptide. These oligonucleotides have been briefly described in the discussion of the first aspect of the invention.

In a sixth aspect, the invention also relates to pharmaceutical compositions which comprise an effective amount of at least one of the anti-beta/A4 oligonucleotides of the present invention together with a pharmaceutically acceptable carrier.

In a seventh aspect, the invention relates to a composition of matter which comprises nerve growth factor and an anti-beta/A4 oligonucleotide.

In an eighth aspect, the invention provides pharmaceutical compositions which comprise nerve growth factor and an anti-beta/A4 oligonucleotide.

In a ninth aspect, the invention teaches a method for measuring effect of APP antisense compounds on Alzheimer amyloid production by propagating a first and a second culture of mammalian cells, such as PC12, which express or preferably overexpress beta/A4; adding labelled methionine to the first and the second cell cultures; incubating the cell cultures for a period of time; collecting supernatant from the first and second cell cultures; candidate APP antisense compound

In a tenth aspect, the invention teaches a method for prevention, treatment and/or reversal of beta/A4 amyloid-induced morphology by administering an effective amount of a pharmaceutical composition which comprise nerve growth factor and an anti-beta/A4 oligonucleotide to a patient in need thereof.

Brief Description of the Figures

Figure 1: shows certain preferred cap structures used in one embodiment of oligonucleotides according to the invention.

Figure 2: shows one form of a self-stabilized oligonucleotide embodiment according to the invention.

Figure 3: shows a second form of a self-stabilized oligonucleotide embodiment according to the invention.

Figure 4: shows a schematic representation of Min vectors containing amyloid cDNA. The insert segment shown harbors the ß/A4 peptide (black vertical box), the rest of the APP coding sequence (open vertical box) and noncoding region (thin vertical line).

Figures 5A, 5B, and 5C: show immunostaining patterns for untransfected cells (FIG. 5A), cells containing the transforming vector without an amyloid insert (FIG. 5B) and cells transfected with vector DNA coding for the region from A4 to the C-terminus of APP (FIG. 5C).

Figures 6A, 6B, 6C, and 6D: show light micrographs of ß/A4 transfected AC126 (FIG. 6C) and AC127 (FIG. 6D) cells compared with untransfected cells (FIG. 6A) or cells transfected with insertless vector (FIG. 6B).

Figure 7: shows a graphical representation of neurite length and cell size for cells transfected with insertless vector (V120) and ß/A4 transfected cells (AC126 and AC127).

Figures 8A and 8B: show electron micrographs of untransfected (FIG. 8A) and ß/A4 transfected (FIG. 8B) PC12 cells.

Figures 9A, 9B, 9C, and 9D: show electron micrographs of membrane processes from untransfected cells (Panel A), insertless vector transfected cells (FIG. 9B), and ß/A4 transfected AC126 (FIG. 9C) and AC127 (FIG. 9D) cells.

Figure 10: shows a graphical representation of the frequency of microvilli and/or bleb-like structures in membranes of untransfected (NN), insertless vector transfected (V120) and ß/A4 transfected (AC126 and AC127) cells.

Figures 11A, 11B, 11C, and 11D: show electron micrographs of untransfected cells (FIG. 11A), cells transfected with insertless vector (FIG. 11B), and ß/A4 transfected AC126 (FIG. 11C) and AC127 cells (FIG. 11D).

Figures 12A and 12B: show electron micrographs of immunostained cells using anti-ribonuclease inhibitor protein antibody (FIG. 12A) or anti-ß/A4 antibody (FIG. 12B).

Figures 13A and 13B: depict the cDNA sequence of APP [SEQ ID NO. 12] disclosed by Kang *et al., Nature 325*:733-736 (1987), from which anti-beta/A4 oligonucleotides can be constructed which are complementary to the corresponding mRNA sequence. The initiation codon begins with nucleotide 147. The coding sequence of the β/A4 protein extends from nucleotide 1935 through 2060. The APP coding sequence ends at nucleotide 2231.

Figures 14A and 14B: depict immunostained AC127 cells that have been grown in the absence (Fig. 14A) or presence (Fig. 14B) of the antisense oligonucleotide having SEQ ID NO. 1.

Figures 15A, 15b and 15C: depict PC12 cell morphology after addition of antisense oligonucleotide. PC12 cells were incubated for one week with varying concentrations of the oligo-1 antisense oligonucleotide complementary to the 5′ end region of APP mRNA. (FIG. 15A) Untreated cells. (FIG. 15B): 10 μg/ml oligo-1 was added. (FIG. 15C) 20 μg/ml oligo-1 was added. Cells were fixed and stained with 0.1% Coomassie blue. Bar = 50 μm.

Figure 16: depicts quantitation of antisense oligonucleotide effect on cellular surface area. The oligo-1 antisense oligonucleotide and the unrelated oligo-2 were added to PC12 cell cultures at 0, 10, and 20 μg/ml for one week. The cells were subsequently fixed and stained with 0.1% Coomassie blue prior to image analysis measurements of cell body area. A significant decline in surface area was observed after oligo-1 treatment at both 10 and 20 μg/ml ($p < 0.01$). The effect of oligo-2 was not significant. 150 cells of each type were counted.

Figures 17A, 17B and 17C: depict Western blots of protein extracts from PC12 cells treated for 10 days with 10 μg/ml of oligo-1. Immunostaining used anti-APP antibody. (FIG. 17A) Protein extracts of untreated cells demonstrating major APP bands of 120-150 kDa. Molecular weight markers are indicated. (FIG. 17B) Treatment with oligo-1 at 10 μg/ml produced a 33% decline in APP levels as compared with untreated controls

and expressed relative to total protein loaded. (FIG. 17C) Treatment with 15 μg/ml caused the APP levels to decrease by 60%. Quantitation was carried out by image analyses.

Figure 18: depicts effect of oligo-1 on PC12 cell area after pre-treatment with NGF. Cells were treated with 100 ng/ml of NGF for 48 hours and subsequently exposed to oligo-1 at a concentration of 15 μg/ml for 0, 1 and 2 days, as shown in the figure. After initial NGF treatment, the cell body size (indicated as day 0) was significantly greater ($p < 0.05$) than the size of untreated cells that were less than 500 μm^2. Subsequent addition of oligo-1 for one and two days had no apparent effect on the response to NGF. Cells were fixed and stained with 0.1% Coomassie blue prior to image analysis of cell body area. Each bar represents the mean value of at least 100 observations.

Figure 19: depicts effect of oligo-1 on PC12 neurite length after pre-treatment with NGF. Conditions and data analysis were the same as indicated in the legend to Figure 18.

Figure 20: depicts effect of NGF on PC12 cellular area after pre-treatment with oligo-1 or oligo-2. PC12 cells were incubated for two days with oligo-1 at 0, 5, 10, or 15 μg/ml and then were maintained at the same oligonucleotide concentration plus 100 ng/ml of NGF for four additional days. At 15 μg/ml, the effect of oligo-1 on surface area was significant ($p < 0.01$) compared to cells unexposed to antisense oligonucleotide. The same experiment was repeated using oligo-2 at 0 and 15 μg/ml. Cells were fixed and stained with 0.1% Comassie blue and evaluated by image analysis. Each bar represents the mean value of at least 100 measurements.

Figure 21: depicts effect of NGF on PC12 neurite length after pre-treatment with oligo-1 or oligo-2. PC12 cells were incubated for two days in the absence of oligonucleotides (control) or in the presence of oligo-1 or oligo-2 at a concentration of 15 μg/ml, as indicated. Cells were processed and neurite length was measured. The effect of oligo-1 was significant ($p < 0.01$)

compared to cells unexposed to oligo-1. Each bar represents the mean value of at least 100 measurements.

Figures 22A and 22B: depict effect of NGF on PC12 cells pre-treated with antisense oligonucleotide. (FIG. 22A) Cells treated with NGF alone. (FIG. 22B) PC12 cells were incubated for two days with oligo-1 at 15 ng/ml and then were maintained at the same oligonucleotide concentration plus 100 ng/ml of NGF for four additional days. Bar = 50 μm.

Detailed Description of the Preferred Embodiments

The invention relates to the development of therapeutic treatments for Alzheimer's disease (AD) and Down's syndrome (DS). The invention provides potential therapeutic agents that are capable of preventing and reversing deleterious effects that are visited upon cells by ß/A4 amyloid peptide, a peptide that is important to the pathology of AD and DS.
Hence, in the present invention, "treatment" means preventing, reducing and/or reversing.

In a first aspect, the invention provides a method of reversing morphological changes induced upon cells by expression of ß/A4 peptide. In this method according to the invention anti-ß/A4 oligonucleotides are administered to cells that express ß/A4 peptide, and that have undergone morphological changes as a result of their expression of ß/A4 peptide. Such morphological changes include, but are not limited to, cell size enlargement, cellular aggregation, formation of bleb and/or microvilli-like membrane processes, and increases in neurite length.

In the method according to the invention, "anti-ß/A4 oligonucleotides" are defined as those oligonucleotides that have a nucleotide sequence that interacts through specific Watson-Crick or Hoogstein base pairing with a specific complementary nucleic acid sequence involved in the expression of ß/A4 peptide, such that the expression of the ß/A4 peptide is reduced. Preferably, the interaction between the oligonucleotide and the specific nucleic

acid sequence involved in the expression of the ß/A4 peptide is either duplex formation by Watson-Crick base pairing, triplex formation by Hoogstein base pairing, or a combination of these. Preferably, the specific nucleic acid sequence involved in the expression of ß/A4 peptide is a gene or RNA molecule that encodes at least ß/A4 peptide. In this context, the term "gene" describes a structure comprising a promoter, a nucleotide sequence encoding at least ß/A4 peptide, and a passive terminator. In one most preferred embodiment, this gene is the well known APP gene. Similarly, the term "RNA" is intended to encompass nuclear or messenger RNA encoding at least ß/A4 peptide. Preferably, such RNA encodes APP protein. In certain embodiments of the method according to the invention, the oligonucleotides administered to cells will be complementary to a nucleotide sequence comprising the initiation codon from which the ß/A4 peptide is translated. The term "complementary to a nucleotide sequence" means sufficiently complementary to such a sequence as to allow hybridization to that sequence in a cell, i.e., under physiological conditions. As a practical matter, the presence or absence of such hybridization can be assessed by determining whether gene expression is reduced. The term "initiation codon from which ß/A4 peptide is translated" means a translation initiation codon that acts as the beginning codon for translation that produces a polyamino acid product that comprises ß/A4 peptide. In a most preferred embodiment, the initiation codon is the initiation codon for APP. In certain other embodiments, the method according to the invention utilizes administration of oligonucleotides that are complementary to nucleotide sequences that encode ß/A4 peptide. Alternatively, such oligonucleotides may be complementary to a nucleotide sequence that comprises a nucleotide sequence encoding ß/A4 peptide. A preferred example of such latter oligonucleotides is an oligonucleotide that is complementary to a nucleotide sequence that encodes APP. Particular examples of such oligonucleotides include, but are not limited to:

1. [SEQ ID NO. 1] 5'-CCTCTCTGTTTAAAACTTTATCCAT-3';
2. [SEQ ID NO. 2] 5'-TTCATATCCTGAGTCATGTCG-3';
3. [SEQ ID NO. 3] 3'-GTCCCAGCGCTACGACGGGCCAAA-5';
4. [SEQ ID NO. 4] 3'-GTCCCAGCGCTAC-5';
5. [SEQ ID NO. 5] 3'-TACGACGGGCCAAA-5';
6. [SEQ ID NO. 6] 3'-GTCCCAGCGCTACGACGGGCC-5';
7. [SEQ ID NO. 7] 3'-GTCCCAGCGCTACGACGG-5';
8. [SEQ ID NO. 8] 3'-GTCCCAGCGCTACGA-5';
9. [SEQ ID NO. 9] 3'-CCAGCGCTACGACGGGCCAAA-5';
10. [SEQ ID NO. 10] 3'-GCGCTACGACGGGCCAAA-5';
11. [SEQ ID NO. 11] 3'-CTACGACGGGCCAAA-5'; and
12. [SEQ ID NO. 15] 5'-AAACCGGGCAGCATCGCGACCCTG-3'.

Preferred oligonucleotides that are useful in the method according to this aspect of the invention are discussed in greater detail later in a discussion of a third aspect of the invention. Briefly, they are generally more resistant to nucleolytic degradation than conventional oligonucleotide phosphodiesters. In certain preferred embodiments, such oligonucleotides may have any of a variety of modified internucleoside linkages, mixed backbones, nuclease resistant 3' cap structures, integrated triplex-forming structures, or self-stabilized structures, or any combination of these.

The method according to this aspect of the invention is useful for a variety of purposes. In particular, this method can provide information about the dosage or level of expression of ß/A4 peptide that is necessary to produce each of the morphological changes in cells. This can be accomplished by reducing the quantity of oligonucleotide administered to the cells, such that the expression of ß/A4 peptide is only partially reduced. The method can also provide information about the time and sequence of morphological changes induced by ß/A4 peptide, by first fully reversing the changes, then removing the oligonucleotide and observing the timing and sequence of the recurrence of the changes. In applied applications, the method provides information

about the specific nature of the oligonucleotides that are most effective for reversing the morphological changes induced by ß/A4 peptide. The present invention is based upon the discovery that, surprisingly, oligonucleotides can reverse morphological changes induced by ß/A4 peptide. Moreover, however, through examining the dosage requirements and degree of morphology change reversal, the method allows those skilled in the art to determine the most efficacious combination of nucleotide sequence, backbone composition, secondary structure, base modification, etc., for reversal of morphological changes.

In a second aspect, the invention provides a method for reducing ß/A4 peptide expression in an animal, including a human. In the method according to this aspect of the invention, oligonucleotides are administered to an animal which cause the reduction in ß/A4 peptide expression. For administration to a nonhuman animal, the nucleotide sequence of the oligonucleotides may be complementary to the appropriate nonhuman ß/A4 peptide or APP gene. The chemical composition of the oligonucleotides is described in detail in the discussion below of the third aspect of the present invention. These oligonucleotides act in the same manner as described for the method according to the first aspect of the invention. The oligonucleotides may be administered orally, intravenously, intranasally, intraperitoneally, anally, by injection into the cerebrospinal fluid, or by direct injection into the brain. Alternatively, the oligonucleotides of the present invention are compounded as part of an implant comprising a polymeric carrier or capsule which allows for sustained release. Such polymeric carriers are disclosed, for example, in Remington's *Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, Osol (ed.) (1990). In a further embodiment, the oligonucleotides of the present invention may be continuously administered by a pump implant or an external pump.

Preferably, the oligonucleotides are administered at a dosage of from about 1 to about 100 mg/kg of animal body weight.

The anti-beta/A4 oligonucleotides are administered as part of pharmaceutical compositions comprising a pharmaceutically acceptable carrier which may be, for example, physiologic saline or physiologic glucose solution. Aqueous injection suspensions which may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Methods for preparing and administering such pharmaceutical compositions may be found in Remington's *Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, Osol (ed.) (1990).

In initial studies, the oligonucleotides are preferably administered to a nonhuman animal, most preferably a mammal. Such administration provides information about the most efficacious dosage and route of administration for reducing ß/A4 peptide expression in the animal. Subsequently, the oligonucleotides are administered to a human suffering from AD or DS. Such administration is expected to reverse morphological changes induced upon brain cells by the ß/A4 peptide, thus bringing about a therapeutic effect.

In a third aspect, the invention provides anti-ß/A4 oligonucleotides that are useful for reversing the morphological changes that are induced upon cells by ß/A4 peptide. The "anti-ß/A4 oligonucleotides" according to the invention encompass those oligonucleotides that have a nucleotide sequence that interacts with a specific nucleic acid sequence involved in the expression of ß/A4 peptide, such that the expression of the ß/A4 peptide is reduced. Preferably, the interaction between the oligonucleotide and the specific nucleic acid sequence involved in the expression of ß/A4 peptide is either duplex formation by Watson-Crick base pairing, triplex formation by Hoogstein base pairing, or a combination of these. *See*, for example, PCT publication Nos. WO91/06626, WO92/08791, WO92/11390, and WO92/10590. Preferably, the specific nucleic acid sequence involved in the expression of ß/A4 peptide is a gene or RNA molecule that encodes at least ß/A4 peptide. In this context, the term "gene" describes a structure comprising a promoter, a nucleotide sequence encoding at least ß/A4 peptide, and a passive terminator. In one

most preferred embodiment, this gene is the well known APP gene. Similarly, the term "RNA" is intended to encompass nuclear or messenger RNA encoding at least ß/A4 peptide. Preferably, such RNA encodes APP protein. In certain embodiments of this aspect of the invention, the oligonucleotides are complementary to a nucleotide sequence comprising the initiation codon from which ß/A4 peptide is translated. The term "complementary to a nucleotide sequence" means sufficiently complementary to such a sequence as to allow hybridization to that sequence in a cell, i.e., under physiological conditions. As a practical matter, the presence or absence of such hybridization can be assessed by determining whether gene expression is reduced. The term "initiation codon from which ß/A4 peptide is translated" means a translation initiation codon that acts as the beginning codon for translation that produces a polyamino acid product that comprises ß/A4 peptide. In a most preferred embodiment, the initiation codon is the initiation codon for APP. In certain other embodiments, the anti-ß/A4 oligonucleotides are complementary to nucleotide sequences that encode ß/A4 peptide. Alternatively, such oligonucleotides may be complementary to a nucleotide sequence that comprises a nucleotide sequence encoding ß/A4 peptide. A preferred example of such latter oligonucleotides is an oligonucleotide that is complementary to a nucleotide sequence that encodes APP. Such oligonucleotides may comprise about 8 to about 100 nucleotide bases.

Anti-ß/A4 oligonucleotides according to the invention may optionally have additional ribonucleotide, 2'-substituted ribonucleotide, and/or deoxyribonucleotide monomers, any of which are connected together via 5′ to 3′ linkages which may include any of the internucleotide linkages known in the art. Preferably, such modified oligonucleotides may optionally contain phosphodiester, phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate and/or sulfone internucleotide linkages. Those skilled in the art will recognize that the

synthesis of oligonucleotides containing any of these internucleotide linkages is well known to those skilled in the art, as is illustrated by articles by Uhlmann and Peyman, *Chemical Reviews 90*:543-584 (1990) and Schneider and Banner, *Tetrahedron Lett. 31*:335 (1990). Preferably, modified oligonucleotides according to the invention should contain from about 6 to about 100 monomers in total. Such modified oligonucleotides may also optionally contain modified nucleic acid bases and/or sugars, as well as added substituents, such as diamines, cholesteryl or other lipophilic groups.

In one preferred embodiment, anti-ß/A4 modified oligonucleotides according to the invention are in the form of a mixed backbone oligonucleotide having one or more regions of nucleotides connected by phosphorothioate or phosphorodithioate internucleotide linkages ("phosphorothioate or phosphorodithioate region") as well as one or more regions of nucleotides connected by alkylphosphonate or alkylphosphonothioate internucleotide linkages ("alkylphosphonate or alkylphosphonothioate region"). In this embodiment, at least one alkylphosphonate region preferably includes nucleotides at or near the 5′ end and/or the 3′ end of the oligonucleotide. For purposes of the invention, "at or near the 5′ or the 3′ end of the oligonucleotide" means involving at least one nucleotide within about 5 nucleotides from the 5′ or 3′ end of the oligonucleotide. Preferably, the alkylphosphonate or alkylphosphonothioate region comprises from about 2 to about 10 contiguous nucleotides connected by alkylphosphonate linkages. Preferably, the phosphorothioate or phosphorodithioate region comprises at least 3, and up to about 100 contiguous nucleotides connected by phosphorothioate or phosphorodithioate linkages.

Anti-ß/A4 modified oligonucleotides according to this embodiment of the invention are synthesized by solid phase methods, alternating H-phosphonate chemistry and sulfur oxidation for phosphorothioate regions, and alkylphosphonamidate chemistry for alkylphosphonate regions. A preferred H-phosphonate approach is taught by Agrawal *et al.*, U.S. Patent No. 5,149,798, the teachings of which are hereby incorporated by reference.

Alkylphosphonamidite chemistry is well known in the art, as illustrated by Agrawal and Goodchild, *Tetrahedron Lett. 28*:3539-3542 (1987). Synthesis of phosphorodithioate-containing oligonucleotides is also well known in the art, as illustrated by U.S. Patent No. 5,151,510, the teachings of which are hereby incorporated by reference (See also, *e.g.*, Marshall and Caruthers, *Science 259*:1564-1570 (1993) and references cited therein). Finally, synthesis of alkylphosphonothioate-containing oligonucleotides is known in the art, as illustrated by Padmapriya and Agrawal, *Bioorganic & Medicinal Chemistry Letters 3*:761-764 (1993).

In another preferred embodiment, anti-ß/A4 modified oligonucleotides according to the invention are in the form of a hybrid oligonucleotide having regions of deoxyribonucleotides ("deoxyribonucleotide regions") and regions of ribonucleotides or 2'-substituted ribonucleotides ("ribonucleotide regions"). Preferably, from about one to about all of the internucleotide linkages are phosphorothioate or phosphorodithioate linkages. Preferred 2'-substituted ribonucleotides are halo, amino, alkyl, aryl or lower alkyl (1-6 carbon atoms) substituted ribonucleotides, especially 2'-OMe-ribonucleotides. Preferably, at least some of the ribonucleotide regions include nucleotides present at or near the 5′ end and/or the 3′ end of the oligonucleotide. More preferably, the ribonucleotide regions each comprise from about 2 and preferably from about 4 to about 100 contiguous ribonucleotides and/or 2'-substitute oligonucleotides. The deoxyribonucleotide regions are optional, and when present may contain from about 1 to about 100 contiguous deoxyribonucleotides.

Anti-ß/A4 oligonucleotides according to this embodiment of the invention are typically synthesized by solid phase methods, preferably by the phosphoramidite or H-phosphonate approach, using deoxynucleotide H-phosphonates for deoxyribonucleotide regions, and ribonucleotide or 2'-substituted ribonucleotide H-phosphonates for ribonucleotide regions.

In yet another preferred embodiment, anti-ß/A4 oligonucleotides according to the invention are in the form of an oligonucleotide having at its 5′ and/or preferably at its 3′ end a cap structure that confers exonuclease

resistance to the oligonucleotide. Such modified oligonucleotides preferably also have from 1 to about all modified (non-phosphodiester) internucleotide linkage. Preferred cap structures include those shown in Figure 1, as well as lower alkyl (C1-C12) or alcohol groups. Preferred modified internucleotide linkages include phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, bridged phosphorothioate, sulfone, phosphorothioate and phosphorodithioate linkages.

Anti-ß/A4 oligonucleotides according to this embodiment of the invention are synthesized according to procedures well known in the art (see *e.g.*, Uhlmann and Peyman, *Chemical Reviews 90*:543-584 (1990); Schneider and Banner, *Tetrahedron Lett. 31*:335 (1990)). For oligonucleotides having cap structures at the 3′ end, the cap structure is reversibly attached to the solid support and is then coupled to the first nucleotide monomer in the synthesis scheme. For oligonucleotides having cap structures at the 5′ end, the cap structure is coupled to the end of the oligonucleotide after addition of the last nucleotide monomer in the synthesis scheme.

In another preferred embodiment, anti-ß/A4 oligonucleotides are self-stabilized by having a self-complementary region at the 3′ end that hybridizes intramolecularly with the oligonucleotide to form an exonuclease resistant hairpin-like structure. Anti-ß/A4 oligonucleotides according to this embodiment of the invention are generally characterized by having two regions: a target hybridizing region and a self-complementary region. The target hybridizing region has a nucleotide sequence that is complementary to the targets described earlier. Preferably, this region has from about 6 to about 100 nucleotides. One such embodiment of the invention is shown in Figure 2. In this embodiment, the target hybridizing region is shown as connected rectangular squares, and the self-complementary region is shown as connected circles. The complementary nucleic acid sequence in a target influenza messenger RNA molecule is represented by connected diamonds. Hydrogen bonding between nucleotides is indicated by dots. The oligonucleotide is

stabilized, *i.e.*, rendered resistant to exonucleolytic degradation by base-pairing between the target hybridizing region and the self-complementary region and/or by base-pairing between complementary sequences within the self-complementary region. When the oligonucleotide encounters a target nucleic acid molecule having a complementary nucleic acid sequence, base-pairing between the target hybridizing region and the self-complementary region of the oligonucleotide is disrupted and replaced by base-pairing between the target hybridizing region of the oligonucleotide and the complementary nucleic acid sequence of the target nucleic acid molecule. This disruption and replacement of base-pairing takes place because the intermolecular base-paired structure formed by the hybrid between the target nucleic acid sequence and the target hybridizing region is more thermodynamically stable than the intramolecular base-paired structure formed by the self-complementary oligonucleotide.

A second form of an oligonucleotide according to this embodiment of the invention operates in a similar way as the first form, but forms a different structure upon self-complementary base-pairing. This alternative form forms a hammer-like structure as shown in Figure 3. In this form, the self-complementary region contains oligonucleotide sequences that can base pair with other oligonucleotide sequences within the self-complementary region. The self-complementary region may also contain oligonucleotide sequences that are complementary to the target hybridizing region.

The second significant region of self-stabilized oligonucleotides according to the invention is the self-complementary region. The self-complementary region contains oligonucleotide sequences that are complementary to other oligonucleotide sequences within the oligonucleotide. These other oligonucleotide sequences may be within the target hybridizing region or within the self-complementary region, or they may span both regions. The complementary sequences form base pairs, resulting in the formation of a hairpin structure, as shown in Figure 2, or a hammer-like structure, as shown in Figure 3. Either the hairpin structure or the hammer-

like structure can have loops resulting from non-base-paired nucleotides, as shown in Figure 2 for the hairpin structure, or can be devoid of such loops, as shown in Figure 3 for the hammer-like structure. The number of base-pairs to be formed by intramolecular hybridization involving the self-complementary region may vary, but should be adequate to maintain a double-stranded structure so that the 3′ end is not accessible to exonucleases. Generally, about 4 or more base-pairs will be necessary to maintain such a double-stranded structure. In a preferred embodiment, there are about 10 intramolecular base-pairs formed in the self-stabilized oligonucleotide, with the 10 base pairs being consecutive and involving the 3′-most nucleotides. Of course, the intra-molecular base-pairing can be so extensive as to involve every nucleotide of the oligonucleotide. Preferably, this will involve a self-complementary region of about 50 nucleotides or less.

Oligonucleotides according to this embodiment may have from 1 to about all modified internucleotide linkages, as described for the fourth embodiment. Preferably, at least either the target hybridizing region or the self-complementary region, and most preferably both, will contain from about 2 to about all nucleotides being coupled by phosphorothioate and/or phosphorodithioate linkages.

As mentioned before, the amyloid precursor protein (APP) is widely distributed among eukaryotic cells, however, its precise role in cellular functioning is not fully clarified. The APP is a glycoprotein membrane constituent and may facilitate membrane associated functions. The possibility that the APP may play a role in mediating cellular trophic responses was explored. An antisense oligonucleotide was prepared to the 5′ terminus of APP and shown to specifically reduce the level of APP isoforms. In sequential mixing experiments it was observed that the APP antisense oligonucleotide did not significantly modify the trophic response of PC12 cells pre-treated with NGF. However, pre-treatment of cells with the antisense oligonucleotide diminished NGF-induced increases in cellular size and neurite length. These observations suggest that APP may play a role in modulating

the trophic response. The combined use of APP antisense oligonucleotides and neurotrophic agents may find clinical utility in the treatment of Alzheimer-type dementia since it is known that NGF normally causes increases in APP levels.

The above-described oligonucleotides of the present invention are preferably used in conjunction with nerve growth factors (NGF) to prevent and/or reverse the morphological changes brought about by amyloid deposits *in vivo* or *in vitro*. Other growth factors such as epidermal growth factor (EGF) can also be used as long as the cells used have receptors for that particular growth factor and can be stimulated to grow or become differentiated. Further, the source of the growth factor, including, NGF, can be from any mammal, including mouse and human. The oligonucleotides are preferably added after addition/administration of NGF in order to stimulate normal neuronal cell growth and proliferation.

As reported in L. Olson, *Experimental Neurology 124*:5-15 (1993), based on background information from intracerebral infusion of NGF in parkinsonian patients, attempting to support intraputaminal chromaffin tissue grafts, a study was initiated using a radio-controlled fully implantable pumping device delivering NGF to the lateral ventricle. Several transient or more long-lasting improvements were noted in the pilot case. These involved increases of blood flow and nicotine binding as evaluated by positron-emission tomography as well as improvement of the EEG and certain psychological tests, tapping verbal episodic memory. Nicotinic receptor sites are characteristically lost in Alzheimer brain tissue as demonstrated in autopsy material. Therefore, it is quite interesting that the [^{11}C]-nicotine uptake and binding were increased in the Alzheimer's patient in cortical areas known to be afflicted in Alzheimer's disease. An interesting surprise was the striking increase in cerebral blood flow demonstrated by PET using labeled butanol. Moreover, the EEG readings indicated improvements that were considerably longer lasting than those seen for blood flow measurements, They also provide another independent indication of a positive change in the patient.

The fact that a partial normalization of the EEG was still noticeable 1 year after cessation of the NGF infusion is also encouraging.

The PET measurements of both blood flow and nicotine binding, the EEG changes, some of the cognitive tests, as well as reports from the spouse of partially recovered verbal communication and activities of daily living suggest positive transient changes. The fact that several of these variables returned to pre-treatment levels in follow-up assessments suggest that they were causally related to the NGF infusion.

The results from this first Alzheimer patient who received NGF and the Parkinsonian patients who received intraputaminal adrenal medullary tissue autografts supported by intraputaminal NGF infusion suggest that delivery of highly purified mouse beta-NGF to the human brain, either into the center of the putamen or into a lateral ventricle over prolonged periods of time, is reasonably well tolerated.

Further, in the case of the Alzheimer patient, the measurement of NGF amount and bioactivity in lumbar CSF samples indicated that intraventricular infusion leads to effective levels of NGF in the CSF also as remote from the infusion site as the lumbar level. Also, blood samples from four patients prior to, during, and after NGF treatment have suggested that NGF from these two intracerebral routs does not reach the general circulation in any appreciable amounts, and also that significant amounts of antibodies against NGF have not been formed.

Hence, another aspect of the invention relates to a composition of matter which comprises nerve growth factor and an anti-beta/A4 oligonucleotide. The anti-beta/A4 oligonucleotide may be complementary to an initiation codon from which beta/A4 peptide is translated. Moreover, the oligonucleotide may be an RNA, which is preferably complementary to a region of beta/A4 RNA. RNA and DNA oligonucleotides are expected to bind to beta/A4 mRNA (e.g., to the exposed nucleotides such as those which are part of a hairpin loop in the beta/A4 mRNA) and/or DNA encoding APP, thereby preventing their transcription and/or translation, respectively. Such

composition of matter is useful, for example, in analytical assays to determine the ideal combination of a growth factor and an antisense oligonucleotide in treatment of degenerative diseases (e.g., AD and DS) which are related to overproduction of native cellular proteins. In such diseases, application of the appropriate growth factor will promote reconstruction of the deteriorated tissue while the antisense oligos regulate the level of production of the native protein. In particular, the assay provide information about the dosage or level of expression of β/A4 peptide and dosage and kind of growth factor, *e.g.* NGF or EGF, that is necessary to reverse each of the abnormal morphological changes in cells which display morphology characteristic of the abnormal neuronal cells of AD patients. Another aspect of the invention relates to kits for a pharmaceutical administration to patients in need thereof which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

(a) the first container means contains a beta/A4 antisense oligonucleotide; and

(b) a second container means contains a growth factor.

The antisense oligonucleotides of the present invention are present in the kits as a solution in an aqueous buffer or a physiological solution. The preferred growth factor of the present invention is a nerve growth factor, however, other growth factors may be applied, such as epidermal growth factor. The growth factor, *e.g.*, a nerve growth factor, may be present as a solution in an aqueous buffer or a physiological solution. The growth factor, *e.g.*, a nerve growth factor, may be present in lyophilized form.

Moreover, the kits may further comprise

(c) one or more container means containing a different growth factor than that used in step (b), above, which may be present as a solution in an aqueous buffer or a physiological solution.

Physiological solution in the present invention means a solution which comprises compounds at physiological pH, about 7.4, which closely represents a bodily or biological fluid, such as CSF, blood, plasma, et cetera.

A further aspect of the invention relates to a pharmaceutical composition which comprises a composition of matter comprising a naturally produced or a synthetically made growth factor and an anti-beta/A4 oligonucleotide in a pharmaceutically acceptable carrier. When used in treatment of AD or DS, the growth factor is preferably a nerve growth factor but it may be any growth factor for which there is a receptor on the neuronal cells of the animal or human patient, and is capable of stimulating the neuronal cells to grow and differentiate. An example of such other growth factors is epidermal growth factor (EGF).

Increasing NGF tonus in AD and DS patients can be achieved in many different ways. In some of these methods both the NGF and the antisense oligos can be delivered to the patient via the same route. Other methods allow increasing NGF tonus independent of the method used to deliver the oligos to the patient. There are a number of reports that suggest that intracerebralventricular (icv) administration of antisense compounds can have effects in areas of the brain some distance away from the site of infusion. *See* for examole, Wahlestedt *et al.*, *Science 259*:528-531 (1993); Sakai *et al.*, *J. Neurochem. 62*:2053-2056 (1994); Zhou *et al.*, *J. Pharmacol. Exp. Ther. 268*:1015-1023 (1994).

1. Intracerebral infusion. The most direct approach is perhaps to deliver purified NGF and/or oligos directly into the central nervous system. NGF does not pass the blood-brain barrier and must therefore be delivered into brain parenchyma or CSF. Chronic infusion of NGF has been tried clinically both in Parkinson's disease and in Alzheimer's disease.

2. Slow-release biodegradable implants. As an alternative to chronic infusion, NGF and/or the oligos may also be incorporated in biodegradable polymer capsules or microspheres, thus providing an implantable slow-release source (Camarata *et al.*, *Neurosurgery 30*:313-319 (1992); Powell *et al.*, *Brain Res. 515*:309-311 (1990)). This technique may prove useful when a local source of NGF and/or oligos is needed in the brain for a limited period of time.

3. *Carrier-mediated transport across the blood-brain barrier.* A third, very promising, method is based on coupling NGF and/or the oligos to a carrier that enables transfer across the blood-brain barrier. Thus it has recently been demonstrated that when NGF is coupled to an antibody to the transferrin receptor, it can be given as an intravenous injection, cross the blood-brain barrier, and retain full biological activity (Friden *et al.*, *Science 259*:373-377 (1993); Cotten *et al.*, *PNAS USA 87*:4033-4037 (1990); Cotten *et al.*, *PNAS USA 89*:6094-6098 (1992); Curiel *et al.*, *PNAS USA 88*:8850-8854 (1991); Wagner *et al.*, *PNAS USA 87*:3410-3414 (1990); Wagner *et al.*, *PNAS USA 88*:4255-4259 (1991); Wagner *et al.*, *Bioconjugate Chem. 2*:226-231 (1991)). Since transferrin receptors are rich on CNS blood vessels, this technique will concentrate NGF and/or the oligos in brain vs. periphery following an i.v. injection.

4. *Grafting NGF-producing cells.* A fourth approach is based on transplanting cells capable of NGF synthesis to intracerebral sites. One might take advantage either of cells that normally produce NGF such as Schwann cells or mouse submaxillary gland cells (Springer *et al.*, *Prog. Brain Res. 78*:401-407 (1988); Springer *et al.*, *J. Neurosci. Res. 19*:291-296 (1988)) or, alternatively, of cells that have been genetically modified to produce and secrete large amounts of NGF. This latter technique has been demonstrated to be efficacious in animals (Ernfors *et al.*, *Proc. Natl. Acad. Sci. USA 86*:4756-4760 (1989); Rosenberg *et al.*, *Science 242*:1575-1578 (1988); Strömberg *et al.*, *J. Neurosci. Res. 25*:405-411 (1990)). While established cell lines have several problems associated with their use, such as the risk of tumor formation and/or the risk of down-regulation of the NGF production, these problems may be overcome by using primary cell lines (Kawaja *et al.*, *J. Neurosci. 12*:2849-2864 (1992)), preferably from the patient who needs treatment, and perhaps by inserting additional genes enabling some control over the NGF production.

5. *Direct gene transfer to the brain.* An interesting alternative to cell transfer to the brain is transfer of only the genes necessary to produce

NGF. Several interesting current approaches suggest that it should indeed be possible to transfect nondividing neuronal and/or gland cells to achieve long-lasting increased NGF levels (Le Gal La Salle *et al.*, *Science 259*:988-990 (1993)).

6. Developing NGF receptor agonists. Knowledge about the neurotrophin receptors is rapidly increasing (Ebendal, T., *J. Neurosci. Res. 32*:461-470 (1992); Ebendal *et al.*, in *Plasticity and Regeneration in the Nervous System* (P. Timiras and A. Privat, Eds.), Plenum, New York). Such knowledge paired with ongoing studies of the tertiary structure of NGF (McDonald *et al.*, *Nature 354*:411 (1991)) and the importance of various domains of the molecule for receptor binding and activation (Ibáñez *et al.*, *Cell 69*:329-341 (1992); Ibáñez *et al.*, *EMBO J. 10*:2105-2110 (1991); Ibáñez *et al.*,*EMBO J. 12*:2281-2293 (1993)) suggests that it might become possible to develop low-molecular-weight agonists capable of passing the blood-brain barrier and exerting NGF-like effects.

7. Controlling endogenous NGF production. Finally, as we gain a better understanding of the control of endogenous NGF synthesis storage and release, pharmacological treatment that could enhance endogenous NGF availability might be envisioned.

Another aspect of the invention, relates to a method for treatment of beta/A4 amyloid-induced abnormal morphology (amyloidosis) which comprises administering an effective amount of a pharmaceutical composition discussed above to a patient in need thereof.

Amyloidosis or beta/A4 amyloid-induced abnormal morphology, as is commonly known in the art and intended in the present specification, refers to the pathogenic condition in humans and other animals which is characterized by formation of Aβ amyloid in neural tissue such as brain.

Yet another aspect of the invention relates to an assay for screening candidate antisense oligonucleotides effective in treatment of deleterious effects that are visited upon cells by ß/A4 amyloid peptide, which comprises

(a) plating several containers of mammalian neuronal cell cultures;

(b) making test samples by adding various concentrations of different beta/A4 antisense oligos to several containers;

(c) making control samples by adding either no oligos or non-sense oligos to several containers;

(d) incubating the test samples and the controls with labelled methionine about 6 to about 24 hours;

(e) collecting supernatant from each container;

(f) contacting the supernatant from each container with protein A sepharose (PAS) to form a PAS-amyloid complex;

(g) contacting the PAS-amyloid complex with an antibody to beta-amyloid to form an PAS-amyloid-antibody complex;

(h) separating the PAS-amyloid-antibody on an acrylamide gel by electrophoresis to form bands of PAS-amyloid-antibody complex corresponding to each cell culture;

(i) determining levels of 4.3 KD beta-amyloid present in the bands by densitometry;

(j) comparing the levels of 4.3 KD beta-amyloid present in test samples with the control samples thereby determining effectiveness of the beta/A4 antisense oligos in reducing production of amyloid in said cells;

(k) selecting those oligonucleotides from step (j) which are most effective in reducing amyloid production;

(l) determining effect of addition of said oligonucleotides, which were selected in step (j), to mammalian neuronal cells treated with a growth factor, which stimulates growth and differentiation of said neuronal cells; and

(m) selecting those oligonucleotides from step (l) which do not inhibit trophic effect of said growth factor.

Labelled methionine as used in the present specification refers to methionine that is bound in any form to a substance that can be detected using

any of several methods known in the. Examples of such labelled methionine are radiolabelled methionine, such as ^{35}S-methionine, or biotinylated methionine.

Complex as used in PAS-amyloid complex or PAS-amyloid-antibody complex means that these molecules are held together by any form of bond possible.

Those skilled in the art will recognize that the features of the various preferred embodiments described above can be combined to produce additional embodiments that may have even greater anti-ß/A4 effect.

Anti-ß/A4 oligonucleotides according to the invention are useful for a variety of purposes. First, they are useful for reversing morphological changes in cells *in vitro* that are caused by expression of ß/A4 peptide. Second, they are useful for examining the effect of reduced ß/A4 peptide expression in animals, including humans. Third, they are useful for conducting clinical trials designed to obtain marketing approval for such oligonucleotides as therapeutic agents for Alzheimer's disease. Forth, they are useful for treating patients suffering from Alzheimer's disease and/or preventing or delaying the onset of the disease. Finally, they are useful for treating patients which are suffering from DS.

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not intended to be limiting in nature. The work described in Examples 1 and 2 below has previously been published in *Proc. Natl. Acad. Sci. USA* 86:337-341 (1989) and *Brain Research* 599:64-72 (1992).

EXAMPLES

Materials And Methods

Unless otherwise indicated, the following materials and methods were used in carrying out the exemplary embodiments of the invention taught in the Examples.

Propagation of cells

PC12 cells were maintained in tissue culture flasks (Becton Dickson, Franklin Lakes, NJ) at 37°C in 5.0% CO_2 in Dulbecco's modified eagle medium (DMEM; GIBCO, BRL, Grand Island, NY), with 2.0 mM (350.4 mg in 0.85% NaCl final concentration) L-glutamine (GIBCO, BRL, Grand Island, NY), 1.2 mM final concentration sodium pyruvate (Sigma, St. Louis, MO), 100U penicillin/mL with 120 μg/mL streptomycin in 0.9% NaCl (Sigma, St. Louis, MO), 10% calf serum, 5% fetal bovine serum, and 20 μg/mL of Gentamycin (Sigma, St. Louis, MO). Upon confluency, the cells were trypsinized using 0.25% trypsin in Hank's balanced salt solution and split into two flasks. PC12 cells were frozen at 1 x 10^6 cells/mL Eppendorf tube in cold 10% dimethylsulfoxide (DMSO) and stored in liquid nitrogen to be brought up as needed. Cell counts were performed using a hemocytometer. Cell viability was determined using trypan blue.

Quantitation of cell morphological features

Cell size and length of neurites were determined at the light microscope level using an image analysis measurement system containing microdensitometry computer software (Bioquant, R&M Biometrics, Inc., Nashville, TN). Cells were grown for 48 hours on glass chambered slides and were rinsed in Hanks buffered saline before being fixed in 4% paraformaldehyde for 30 mins. Following rinsing, cells were stained with Coomassie blue for 5 min. Slides were then rinsed, dried and coverslipped. Fields of cells were examined with a Leitz microscope at 40X. Using the image measurement system, the boundary of the cell body was outlined and the mean area of the cell body was calculated. The number of cells measured in individual experiments is indicated in the legends to figures.

Phosphorothioate oligonucleotides

Oligonucleotide phosphorothioates (PS-oligos) were synthesized on a 10 μmole scale using a phosphoramidite approach on an automated DNA synthesizer (model 8700, Millipore, Milford). PS-oligos were purified by the same procedures as reported earlier (Agarwal *et al., Proc. Natl. Acad. Sci. USA 88*:7595-7599 (1991)). Two 24-mer PS-oligos were synthesized: oligo-1: 5'-AAACCGGGCAGCATCGCGACCCTG-3' (SEQ. ID NO:15), which is complementary to the APP ribosomal binding site; and, oligo-2: 5'-ACACAGCGCGTACGACGACGCGCT-3' (SEQ. ID NO:16) which is a control random antisense oligonucleotide. Lyophilized antisense oligonucleotides were dissolved in sterile water to prepare stock solutions which were stored at -70°. Prior to use aliquots were stored at 4°C and brought to room temperature. The antisense oligonucleotides were added at a final concentration of 5-50 μg/mL depending upon the experimental conditions (see Results). Oligonucleotides were added to fresh culture medium every three days. Cells were cultured on 24-well polystyrene plates at 5 x 10^3 cells/well.

SDS polyacrylamide gel electrophoresis

For SDS PAGE, cells were plated on 3.5 cm 6-well polystyrene plates (Becton Dickson, Lincoln Park, NJ) at a concentration of 5 x 10^5 cells/well. They were rinsed with serum-free media and a solution of 1.2 mM calcium, 1.0 mM magnesium, and 0.01 M Hepes in warmed HBSS at a pH of 7.2. 1000 μl of a 2% SDS solution was placed into each well and allowed to incubate for 30 minutes at room temperature. The lysed cell solution was pipetted into 1.5 ml microcentrifuge tubes. The tubes were boiled for 5 minutes and allowed to cool to room temperature. The cell lysates were stored at 4°C until used further. A bicinchoninic acid protein assay (Deutscher, M.P., "General Methods for Handling Proteins and Enzymes," in

Methods in Enzymology, vol. 182, 60-62 (1990) was used to determine the protein content prior to loading. The lysates were applied to 7.5% gels. Approximately 70 μg of protein was loaded per well in sample buffer (Schagger and Von Jagow, 1987) for a total volume of 70 μl/lane. Gels were run overnight at 15 mA/gel.

Western blots

The gel was transferred onto a Immobilon P, PVDF membrane (Millipore Corp., Bedford, MA) in a TE series Transphor electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA). The transfer buffer consisted of 0.19 M glycine and methanol in a TRIS base buffer, pH 6.8. The proteins were transferred at 1000 mA for 3 h. The membrane was then stained with 0.2% Ponceau to determine the total protein content on each lane. The staining density was determined using computer image analysis (see below). The membrane was blocked with a 10% solution of dry milk for 30 minutes and immunostained with an anti-APP primary monoclonal antibody (Ab) at 1 μg/mL (Boehringer Mannheim) overnight at room temperature. The primary Ab was then removed and the membrane was washed three times in TBST (20 mM Tris, 0.3M NaCl, 0.1% Triton-X 100, pH of 7.2). The secondary antibody was peroxidase conjugated goat anti-mouse IgG whole molecule (Cappel, West Chester, PA) diluted 1:1000 in ICC (2% BSA, 0.3M NaCl, 20 mM Tris, 0.1% Triton-X 100, and 0.1% thimerosal. After one hour of incubation at room temperature with the secondary Ab, the blot was washed four times for fifteen minutes each with TBST. The visualization step used 0.5 μl 30% H_2O_2/mL and 0.5 mg/mL diaminobenzadine (Sigma, St. Louis, MO) in 1 mg/mL final concentration imidazole buffer, pH 7.0 (Sigma, St. Louis, MO). The membrane was rinsed well with water several times. The blot was then scanned using an image processor and the optical density of antibody staining was normalized to Ponceau staining.

Image analysis

Cell area was determined by light microscopy using an image analysis measurement system. Cells prepared for image analysis were prefixed in Hanks buffered saline with 5 mM EGTA/well and 200 μL 0.4% glutaraldehyde for five minutes. This was removed and 2% glutaraldehyde was added to fix the cells overnight. The cells were then stained with 0.1% Coomassie blue in 20% MeOH and 1% acetic acid for 20 minutes. For destaining, the Coomassie blue stain was removed and the cells rinsed with 20% MeOH plus 1% acetic acid for two minutes. The cells were rinsed twice in 1% acetic acid and left in 1% acetic acid until analyzed. Fields of cells were examined with a Reichert Biostar inverted microscope. The image analysis system consisted of a high resolution black and white camera (Javelin, model JE7362), Optimas software, ver 4.2 (available from Image Analysis Systems (Woburn, MA)) and an IBM compatible 486 computer. Using the image measurement system, the boundary of the cell body was outlined and the mean area of the cell body was calculated.

Statistical analysis

Mean values along with standard deviation and standard error were computed with a Microsoft Excel program. The mean value of cell body size was computed with spreadsheet software.

Example 1

Development of a PC12 Cell Line that Overexpresses β/A4 Peptide

The initial cloning vehicle was a simian virus 40(SV40)-based vector pKo+RI/ML, composed of PML_2, a derivative of pBR322 (lacking certain prokaryotic sequences poisonous for eukaryotic cell replication), the Lac UV5

promoter of *Escherichia coli*, and SV40 sequences covering the enhancer, origin of replication, early promoter, small tumor (t)/large tumor (T) antigen splice sites, and polyadenylation sites. Modification of the initial vector was carried out to produce three variants, Min+1, Min+2, and Min+3 with three different translational reading frames using the ATG codon of the T/t antigen (see Fig. 4). The starting vector or modified forms were used for experimentation. The precursor to the Min series contained a unique *Pvu* II site (enhancer start) and a *Bam*HI site [poly(A) addition site], both of which were modified to *Xba* I sites by standard techniques.

From an AD brain cDNA expression vector library prepared with bacteriophage λ we obtained an insert, referred to as amy37, that included the A4 sequence and the flanking regions. The Min vector constructs were used for insertion of the *Eco*RI-digested amy37 cDNA fragment in the three transitional reading frames. Vectors were digested with *Eco*RI restriction endonuclease to cleave at the unique *Eco*RI site and with alkaline phosphatase. The λt11-amy37 chimera was digested with *Eco*RI enzyme and the 1.1-kilobase (kb)-long fragment was ligated into the Min vectors by established techniques. The amy37-1.1 chimeric plasmids generated separately in the three reading frames were propagated, then the DNA was isolated, purified and used for transfection experiments. The cell line used for these experiments was PC12, derived from rat adrenal pheochromocytoma.

Conventional permanent transfection experiments were conducted. Integration of the 1.1-kb amyloid cDNA insert was carried out using transfection medium containing 10 μg of vector with amy37-1.1 inserts or a control consisting of vector DNA without an amyloid cDNA insert, 5 μg of PSV_2CAT DNA (the chloramphenicol acetyltransferase gene cloned into an SV40-based plasmid), which carried the gene for neomycin resistance that was sensitive to Geneticin. The various transfectants were selected for survival in the presence of Geneticin (G418, GIBCO) at a concentration of 0.4 g/liter for 6 days and then at 0.3 g/liter for 3 days; the cells were subsequently

maintained at 0.2 g/liter. The cells shown in accompanying figures had undergone at least 20 cell divisions.

DNA was isolated from cells, and Southern blots were prepared. Nytran filters were hybridized overnight at 52°C in hybridization solution containing 3x Denhardt's solution (1x Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) and amy37-1.1 riboprobe at 8 ng/ml (2.5 x 10^6 cpm/ml) that had been denatured by heating at 80°C for 8 min. The riboprobe prepared had a specific activity of 3.1 x 10^8 cpm/μg. The filters were washed twice for 5 min in 2 x SSC (1 X SSC = 0.15 M NaCl and 0.015 M sodium citrate)/0.1% sodium dodecyl sulfate (SDS) at 25°C and then twice for 30 min. in 0.1% SSC/0.1% SDS at 53°C. The filters were air dried and used for autoradiography. ß/A4 C-terminal transfectants exhibited a hybridization signal corresponding to a human ß/A4 DNA fragment of around 1200-1300 b.p., the size of the known *Eco*RI fragment encoding the C-terminal region.

PC12 cells were immunostained before and after transfection with the in-frame vector Min+2amy37-1.1 and the out-of-frame vector Min+3-amy37-1.1. Logarithmic phase cultures of non-transfected cells and those carrying the vector without the A4-coding insert typically exhibited barely detectable antigen levels after application of the anti-A4 mAbs (Figs. 5A and 5B, respectively). However, the in-frame vector produced cells with unusually high levels of reaction product after immunostaining (Fig. 5C). In some instances the antigen appeared concentrated around the periphery or was localized to one end of the cell. Dividing cells exhibited lighter immunostaining with an uneven distribution of reaction product. PC12 cell lines that overexpress the A4 epitope have been propagated in culture for a period of > 2 months. The cell lines examined were designated NN (untransfected PC12 cells), V120 (insertless vector-transfected PC12 cells), AC126 and AC127 (ß/A4 C-terminal peptide expressing PC12 cells).

Example 2

Assessment of Altered Morphology Cells Overexpressing ß/A4 Peptide

Cell size and length of neurites were determined at the light microscope level using an image analysis measurement system containing microdensitometry computer software (Bioquant, R&M Biometrics, Inc., Nashville, TN). Cells were grown for 48 hours on glass chambered slides and were rinsed in Hanks buffered saline before being fixed in 4% paraformaldehyde for 30 min. Following rinsing, cells were stained with Coomassie blue for 5 min. Slides were then rinsed, dried and coverslipped. Fields of cells were examined with a Leitz microscope at 40X. Using the image measurement system, the boundary of the cell body was outlined and the mean area of the cell body was calculated. The neurites from each measured cell were traced and neurite length was calculated. Data was derived from counting 100 V120 cells from two separate experiments and 150 NN, AC126 and AC127 cells from three separate experiments. Data were compared by analysis of variance.

For electron microscopy, cells were fixed with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min, postfixed with OsO_4 for 30 min, dehydrated through graded ethanols, and embedded in Epon on coverslipped glass slides. Sections were cut using an ultra-microtome (60 nm) and placed on grids. The grids were examined with JEOL 1200EX electron microscope at a magnification of 5,000X, 10,000X and 40,000X. For the purposes of quantitating membranous structures, montages of photographs were made with pictures photographed at 5,000X.

Membrane processes were observed that resembled previously described microvilli and blebs. The former appear in electron micrographs as rounded or finger-like membrane limited cytoplasmic protrusions at the cell surface with a diameter ranging from 0.1 to 10 μm. Other processes appeared similar to blebs since they contained ribosomes or endoplasmic reticulum in

the interior. Blebs often appeared as vesicles at the cell surface which is presumed to reflect the section plane of the block.

For immunoelectron microscopy, cells were grown in 35 mm plastic plates. The plates were rinsed briefly with Hank's salt solution and fixed with fresh 4% paraformaldehyde in 0.13 M NaCl, 0.02 M phosphate buffer, pH 7.4 (PBS) for 30 minutes at room temperature. They were rinsed 3 times with PBS for 10 minutes each time.

Cells were immunostained with monoclonal antibodies (IgG) against a synthetic polypeptide with ß/A4 sequence. For comparative studies a second mAb, that lacks affinity for membrane antigens, was used. In this case the mAb was prepared to alkaline ribonuclease inhibitor protein (RIP) (Promega), an intracytoplasmic regulatory protein that functions to stabilize RNA and ribosomes. The supernatant from the hybridoma cell line making anti-RIP antibodies (IgG) served as a negative control for immunocytochemistry when results were compared to anti-ß/A4 immunostaining.

Primary antibody supernatants were diluted 1:5 in immunostaining buffer (2% bovine serum albumin, 0.3 M NaCl, 0.02 M phosphate, pH 7.2, 0.01% Triton X-100). Incubation was overnight at 4°C. The following day cells were washed 3X for 10 minutes with buffer containing 0.3 M NaCl and 20 mM Tris, followed by a 2 hour incubation with 5 μg/ml of biotinylated goat anti-mouse IgG (Jackson Immunoresearch) in immunostaining buffer (without detergent). Cells were washed as before. They were then incubated with streptavidin-horseradish-peroxidase conjugate (Sigma) at 0.25 μg/ml in immunostaining buffer (without detergent) for 2 hours and washed as above. The chromogen used was diaminobenzidine (Sigma) 0.5 mg/ml, imidazole (Sigma) 1 mg/ml, in 100 mM Tris, pH 7.0. Hydrogen peroxide was added just before use at 0.015% (1 μl of 30% H_2O_2/2 ml). The reaction proceeded for 2 minutes at room temperature followed by two rinses with distilled water. The cells were then post-fixed in 2% glutaraldehyde as for routine electron microscopy except that sections that were photographed were not counterstained with lead or uranium salts.

The mean values corresponding to the frequency of membrane processes at the electron microscopy level was compared using ANOVA followed by the Tukey protected T-test.

PC12 cell lines transfected with ß/A4 C-terminal DNA (AC126 and AC127) were observed to be morphologically altered. Compared to untransfected PC12 cells (NN), and cells transfected with vector alone (V120), the ß/A4-positive transfectants were noticeable larger (Fig. 6, Panels C and D) and had a tendency to aggregate at high density.

Quantitative morphometric light microscopic analyses indicated that the numbers of neurites were not elevated in AC 126 and AC127 cells (75% and 85% of NN). By contrast, neurite length was significantly increased in AC126 and AC127 relative to NN (123% and 254%, resp.) as was cellular area (144% and 234%, respectively) (Fig. 7). V120 failed to exhibit increases in the length of neurites or the area of cells (76% and 96%, respectively, the values of NN cells, Fig. 7).

By electron microscopy NN and V120 cells were morphologically dissimilar to ß/A4-positive transfectants. As anticipated from the light level microscopy AC126 and AC127 cells and nuclei appeared larger than controls and contained more extensive and irregular processes along the entire cell surface. Representative examples are shown in Fig. 8. There was no significant difference among the four cell types (NN, V120, AC126, AC127) with respect to the nuclear to cytoplasmic ratio; the nucleus consisted of approximately 29% of the total volume of the cell.

AC126 and AC127 cell lines appeared to contain fewer chromaffin granules throughout the cytoplasm; however, mitochondrial size and density were unchanged in ß/A4-positive transfectants relative to controls. The characteristic shape and well-defined cristae were preserved and no significant differences were observed for the area of the cell occupied by mitochondria. The number of lipofuscin granules was not significantly altered. No differences were observed with respect to ribosomes, polysomes, rough or smooth endoplasmic reticulum, lamellar bodies or Golgi apparatus.

Cell membranes were examined by electron microscopy to assess apparent morphologic modifications in ß/A4 amyloid-positive transfectants relative to controls. Detailed scrutiny of AC126 and AC127 cell lines revealed increased numbers of membranous elaborations that resembled microvilli and blebs (Fig. 9).

Cell surface elaborations in controls and in ß/A4 positive transfectants were quantified and compared. There were significant increases in the frequency of these structures in ß/A4-positive transfectants compared to normal control and V120 cells (Fig. 10). There were no significant differences between V120 and NN cells.

The possible relationship between sites of increased ß/A4 accumulation and the appearance of membrane extensions at the cell surface was examined by electron microscopy of immunostained sections. After application of monoclonal antibodies prepared against ß/A4, electron micrographs of AC126 and AC127 transfectants were contrasted with control cells. The two ß/A4-positive cell lines exhibited increased levels of antigen within the cell body and there was prominent immunostaining along the length of the plasma membrane (Fig. 11). Deposits of the ß/A4 amyloid antigen were also concentrated within membrane processes resembling blebs and microvilli (Fig. 11).

To demonstrate that anti-amyloid mAbs did not label plasma membranes non-specifically, an unrelated control mAb to a cytoplasmic protein was included for comparison. The latter has high affinity for the rat and human ribonuclease inhibitor protein (RIP). Both mAbs were applied to AC126 cells using identical procedures. The anti-RIP mAb lightly labeled cell cytoplasm in a diffuse pattern and failed to detect membranes (Fig. 12A). By contrast, the anti-ß/A4 mAb stained membranes of processes, as before (Fig. 12B), indicating that non-specific binding of IgG did not preferentially occur when using the described immunocytochemical methodology.

These results demonstrate that the PC12 cells were substantially altered by expression of the beta amyloid peptide. They suggest that insertion of the ß/A4 peptide into the cell membrane allows its expansion and acts in concert

with other unidentified factors to allow PC12 cells to enlarge and to form unusually elongated neurites. Finally, they suggest that ß/A4 peptide likely contributes to the increased aggregation of the transfected PC12 cells.

The data for cellular area and neurite length of PC12 cells and AC127 cells averaged over a large number of experiments is shown in Table I, below.

Table I

Alteration of PC12 Cell Morphology by Beta/A4 Peptide

	PC12 Cells	AC127 Cells
Cellular area (μm^2)	274.76±31	642.28±59
% increase	—	234
Neurite length (μm)	10.36±1.21	26.27±3.32
% increase	—	254

Example 3

Reversal of ß/A4 Peptide-Induced Morphological Alteration By Oligonucleotides

Two oligonucleotides were tested for their ability to reverse ß/A4 peptide-induced morphological alterations in PC12 cells. Both of the oligonucleotides tested were oligonucleoside phosphorothioates (all phosphorothioate internucleoside linkages). The first of these oligonucleotides had the nucleotide sequence [SEQ ID NO. 1] 5'-CCTCTCTGTTTAAAACTTTATCCAT-3'. This sequence is complementary to a nucleic acid sequence that includes the initiation codon from which the ß/A4 peptide sequence is translated, in this case encompassing the SV40 T antigen initiation codon. The second oligonucleotide tested had the nucleotide sequence [SEQ ID NO. 2] 5'-TTCATATCCTGAGTCATGTCG-3'. This oligonucleotide is complementary to a portion of the APP coding sequence

(encoding amino acids 601-607), which corresponds to the sequence encoding amino acids 5-11 of the ß/A4 peptide.

In the experiments that follow, cell size and length of neurites were determined at the light microscope level using an image analysis measurement system containing microdensitometry computer software (obtained from Bioquant, R & M Biometrics, Inc., Nashville, TN). In general, quantitative data on cell parameters (volume, length) for comparison purposes could only be obtained by use of the computer based optical imaging system, rather than by a qualitative comparison of photographs.

Initially, data was collected from normal control PC12 cells and compared to AC127 cells that overexpress beta amyloid in order to establish baseline data. In some experiments, it appeared that the increase in cellular area may have preceded the lengthening of neurites. Morphometric measurements were carried out as described above. The control PC12 cells had a cellular area of 274.76 $\pm$ 31 μm^2 and the neurites had a length of 10.36 $\pm$ 1.21 μm. The AC127 cells had a cellular area of 642.28 $\pm$ 59 μm^2 (234% increase in area) and the neurites had a length of 26.27 $\pm$ 3.32 μm (254% increase in length). *See*, Table 1.

In a first set of experiments, AC127 cells, prepared as described in Example 2, were cultured for 8 days in the presence or absence of 50 $\mu g/ml$ of one of the two test oligonucleotides. Morphological points were then compared for the oligonucleotide-treated and untreated cells. Treatment of cells with the oligonucleotide that is complementary to the initiation codon from which ß/A4 peptide is translated [SEQ ID NO. 1] resulted in greatly diminished immune staining for ß/A4 peptide. As shown in Figures 14A and 14B, immunostaining was noticeably darker for the amyloid positive cells that did not receive the antisense oligonucleotide (Fig. 14A) compared to cells that received the oligonucleotide (Fig. 14B). In addition, these treated cells were clearly disaggregated. In an individual experiment, the size of the treated cells diminished from 455.76 $\pm$ 33.11 μm^2 to 299.12 $\pm$ 31.98 μm^2 (average of 50 cells).

In another individual experiment, treatment of cells with the oligonucleotide complementary to the ß/A4 peptide coding sequence [SEQ ID NO. 2] resulted in a reduction in cell area from 751.67 ± 111.35 μm^2 to 286.25 ± 60.55 μm^2 (average of 25 cells).

In a side-by-side comparison, each oligonucleotide was equally effective in reversing morphological changes induced in PC12 cells by ß/A4 peptide, and a combination of the two was similarly effective. Untreated cells had an area of 715.16 ± 66.96 μm^2. Cells treated with the oligonucleotide having SEQ ID NO. 1 had an area of 378.71 ± 36.29 μ^2. Cells treated with the oligonucleotide having SEQ ID NO. 2 had an area of 347.12 ± 35.36 μm^2. Fifty cells were measured in each experiment.

In a further experiment, the effects of a mixture of the two antisense oligonucleotides on the cellular area of amyloid-positive PC12 cells were determined. To the cultured cells were added 0 or 50 μg/ml of a mixture of antisense oligonucleotides having SEQ ID NO. 1 and SEQ ID NO. 2. Cells cultured in the absence of the antisense oligonucleotides had an area of 786.69 ± 68.23 μm^2 compared to 386.55 ± 34.08 μm^2 for cells treated with the mixture. Fifty cells were measured. Thus, the results show that a mixture of the two antisense oligonucleotides significantly reduced the size of the cells.

Next, the effects of the antisense oligonucleotide having SEQ ID NO. 2 on the length of neurites of amyloid-positive PC12 cells were determined. Cells that overexpress beta amyloid extend neurites to lengths that eventually reach nearly twice that of normal control PC12 cells. In this experiment, the effect of the antisense oligonucleotide having SEQ ID NO. 2 was determined on cells having normal length and cells having abnormally long extensions. Normal control PC12 neurites had a length of 10.36 ± 1.21 μm. In a first group, the neurite length of amyloid positive cells was 13.12 ± 1.21 μm compared to 13.31 ± 1.06 μm after treatment with the antisense oligonucleotide. In a second group, the neurite length of amyloid positive cells was 14.29 ± 1.23 μm compared to 11.80 ± 0.94 μm after treatment with the antisense oligonucleotide. In a third group, the neurite length of

amyloid positive cells was 22.66 ± 3.45 μm compared to 8.952 ± 1.14 μm after treatment with the antisense oligonucleotide. This last group of cells had neurites which were abnormally long. These data demonstrate that the antisense oligonucleotide having SEQ ID NO. 2 is effective in reducing the length of neurites in cells that overexpress beta amyloid only when the transfectants spread extensions to a greater than normal length compared to control values.

In a further experiment, the effectiveness of antisense oligonucleotides having the following sequences and which are completely unrelated to the beta amyloid protein were tested:

[SEQ ID NO. 13] 5'-TTGTTGCGCAGCAGCGTCGTC-3'
[SEQ ID NO. 14] 5'-GGCAAGCTTTATTGAGGCTTAAGCA-3'

An equimolar mixture of the two oligonucleotides were employed at a total concentration of 50 μg/ml. The average size of transfected cells prior to treatment was 560.45 μm compared to 379.29 μm after treatment with the mixture of antisense oligonucleotides having SEQ ID NOS. 13 and 14.

Taken all together, these data show that anti-beta/A4 oligonucleotides effectively reduce the increased size of beta positive cells by approximately 50% The resultant treated cells were, on the average, no more than 18% larger than normal control PC12 cells. By contrast, the oligonucleotides which are unrelated to beta amyloid regulation reduced the size of beta positive PC12 cells by 32% The resultant cells were, on the average, 28% larger than normal control PC12 cells. Thus, antisense oligonucleotides that are complementary to either the initiation codon from which ß/A4 peptide is translated or a nucleotide sequence encoding ß/A4 peptide are capable of reversing morphological changes that have been wrought upon cells by ß/A4 peptide.

Example 4

Morphology of PC12 cells treated with antisense oligonucleotide

PC12 cells treated for one week with 10 or 20 μg/ml of the oligo-1 antisense oligonucleotide complementary to the 5′ end of APP were morphologically assessed by light microscopy. Addition of the antisense oligonucleotide caused an apparent decrease in cell body size (FIGs. 15B, 15C) compared with normal control PC12 cells (FIG. 15A) and cells treated with the unrelated random oligonucleotide, oligo-2. Cells remained viable and morphology remained otherwise preserved.

The apparent decrease in cellular area was confirmed by quantitative morphometry of untreated and treated PC12 cells. As indicated in FIG. 16, cells incubated for one week in the presence of oligo-1 at 10 μg/ml showed a significant decrease in cell surface area; and, there was a further decline in area at 20 μg/ml of antisense oligonucleotide as compared with untreated cells. Addition of oligo-2 failed to produce a significant decrease in cell surface area.

Example 5

APP levels after treatment with antisense oligonucleotide

Detergent-extracted protein from normal control PC12 cells and cells that were treated with PS-oligos was separated on denaturing SDS gels and subjected to immunostaining with anti-APP antibody. On immunoblots APP appeared as two major bands that migrated in the 120-150 kDa size range (FIG. 17A). PC12 cells treated for 10 days with 10 μg/ml of the oligo-1 showed a 33% decline in detergent-extracted APP levels (FIG. 17B) as compared with untreated control cells. At 15 μg/ml of antisense oligonucleotide there was a 60% decrease in extracted APP (FIG. 17C). The

unrelated oligo-2 antisense oligonucleotide at the same concentration had no significant effect. There was no significant difference in levels of tyrosine hydroxylase between PS-oligo-treated and untreated cells on western blots immunostained for tyrosine hydroxylase.

Example 6

Effect of addition of antisense oligonucleotide to NGF-treated PC12 Cells

As expected PC12 cells treated with 100 μg/ml of NGF had a significant trophic response. Cellular area increased from less than 500 μm^2 to greater than 1300 μm^2 over a three day period and they remained more than two-fold larger than unstimulated cells. During the same time interval neurite length increased to approximately 100 μm.

The potential effect of the APP antisense oligonucleotide on PC12 cells pre-treated with NGF was evaluated. PC12 cells exposed to NGF for 48 hours became differentiated and were not significantly affected by the subsequent addition of oligo-1 at 15 μg/ml. The cellular area (FIG. 18) and the neurite length (FIG. 19) increased and reached levels similar to NGF-treated cells that were unexposed to the antisense oligonucleotide. The inability of oligo-1 to hinder the trophic response of PC12 cells was similar to results with oligo-2 (FIG. 19).

Example 7

Effect of NGF addition on PC12 Cells treated with antisense oligonucleotide

PC12 cells were continuously cultured in the presence of oligo-1, at various concentrations, for six days. After day two, cells were also exposed to NGF for the remaining four days at which point morphologic measurements were made. As shown in FIGs. 20 and 21, increasing concentrations of APP

antisense oligonucleotide had increasing capacity to hinder the trophic response to NGF. By contrast, under the same conditions the random sequence oligo-2 had no significant effect. Light microscopy verified that except for size and neuritic length, PC12 cells exposed to oligo-1 remained morphologically normal without apparent deleterious effects to cell vitality (FIGs. 22A and 22B).

In Examples 4-7, the contribution of APP to the effects of a neurotrophic agent by application of an antisense oligonucleotide were examined. Earlier reports indicated a relationship between NGF and the redistribution and release of APP from cultured cells (Fukuyama *et al., Molec. Brain Res. 17*:17-22 (1993); Mobley *et al., Proc. Natl. Acad. Sci. 85*:9811-9815 (1988); Refolo *et al., Biochem. Biophys. Res. Commun. 164*:664-670 (1989); Schubert *et al., Neuron. 3*:689-694 (1989)). However previous studies did not examine a mechanistic linkage between APP levels and the trophic response to NGF.

The mechanism by which regulation of APP levels is associated with the maintenance of PC12 cell surface area and neurite length is not known. APP has been implicated in cell adhesion both directly, through molecular binding studies (Ghiso *et al., J. Biochem. 288*:1053-1059 (1992); Kibbey *et al., Proc. Natl. Acad. Sci. USA 90*:336-342 (1993); Maestre *et al., Neuroscience 18*:1437 [abs. 60.1], 1437 (1992); Schubert *et al., Neuron. 3*:689-694 (1989)), and indirectly, by means of anti-APP antibodies (Breen *et al., J. Neurosci. Res. 28*:90-100 (1991); Chen & Yankner, *Neurosci. Lett. 125*:223-226 (1991)). Thus, the oligo-1-induced decline in cell surface area may be related to decreased cellular adhesion. Decreased surface attachment leading to a more rounded cellular morphology may contribute to the apparent reduction in surface area. Alternatively, maintenance of cell size may be related to possible stimulatory effects of APP, which is known to be secreted by PC12 cells (Schubert *et al., Neuron. 3*:689-694 (1989); Refolo *et al., Biochem. Biophys. Res. Commun. 164*:664-670 (1989)). Treatment with oligo-1 may have resulted in decreased secretion of APP and lowered levels

in the tissue culture medium. It is not known whether modulation of the level of secreted APP is important for cell attachment and vitality. In an earlier study, it was observed that conditioned medium from PC12 cells that overexpress the C-terminal region of APP has a trophic effect on normal PC12 cells.

The possibility that the activity of oligo-1 is attributable to non-specific effects is unsupported by results derived from application of a random PS-oligo. Oligo-2 had an insignificant effect on PC12 morphology and trophic response to NGF. Further evidence that oligo-1 did not mediate non-specific cellular consequences was the observation that the effects of oligo-1 were concentration-dependent in the assay systems employed in the present study.

The differentiated state of PC12 cells in response to NGF was unaffected by the subsequent addition of the antisense oligonucleotide. By contrast pre-treatment of cells with oligo-1 modified the subsequent trophic response to NGF. These observations suggest that APP levels may be important for the early stages of differentiation. In PC12 cells treated with NGF, the 695 isoform of APP increases (Fukuyama *et al., Molec. Brain Res. 17*:17-22 (1993)). APP also redistributes from cytoplasm, in the non-differentiated state, to growth cones, processes and cytoplasm after NGF treatment. Secretion of APP was increased immediately after exposure to NGF (Fukuyama *et al., Molec. Brain Res. 17*:17-22 (1993)). The antisense oligonucleotide-induced reduction of APP levels observed in the present study may be sufficient to directly modulate certain of the cellular effects of NGF. Alternatively, or concurrently, reduction of APP may cause a structural alteration in the plasma membrane sufficient to modify NGF receptor activation.

The potential value of NGF for the treatment of AD has been discussed from various perspectives (Mobley, W.C., *Neurobiol. Aging 10*:578-580 (1989); Olson L., *Exp. Neurol. 124*:5-15 (1993)) and NGF has begun to be applied clinically (Seiger *et al., Behav. Brain Res. 57*:255-261 (1993)). However, a potential encumbrance to this therapeutic approach is suggested

by observations that NGF induces production of APP (Fukuyama *et al., Molec. Brain Res. 17*:17-22 (1993); Mobley *et al., Proc. Natl. Acad. Sci. 85*:9811-9815 (1988); Refolo *et al., Biochem. Biophys. Res. Commun. 164*:664-670 (1989)), which may cause further trophic stimulation or non-specific effects. It is not known whether the NGF-induced elevation of APP contributes to the amyloidosis that is characteristic of AD. However, it has been reported that AD is characterized by increased, rather than decreased levels of cortical NGF (Crutcher *et al., Detection of NGF-Like Activity in Human Brain Tissue: Increased Levels in Alzheimer's Disease* (1993)). The present studies indicate that APP levels can be regulated with specificity by an antisense oligonucleotide. When viewed in terms of the potential benefits of proposed neurotrophic treatments for AD, the possibility arises that the sequential application of growth factors followed by antisense oligonucleotides designed to regulate APP levels may find clinical application in the treatment of Alzheimer's disease.

Example 8

Procedure for measuring effect of APP antisense compounds on Alzheimer amyloid production

Materials and Methods

PC12 (any neuronal cell lines may be used) cells are propagated as described by Greene & Tischler, *Proc. Natl. Acad. Sci USA 73*:2424-2428 (1976) with the following exceptions: Dulbecco's modified Eagle's media (DMEM) with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 units/ml penicillin, 10% calf serum, 5% fetal bovine serum. When added, NGF (2.5S or preferably, 7S mouse, sigma; NGF from rat (mouse or human NGF can also be used) is used at 100 ng/ml (50-200 ng)

after preculturing for 48 hours and renewed at 3 day (2-4 days) intervals. Cultures are kept at 37°C (34-38°C), 5% CO_2 in a humidified incubator.

Cells are plated at 50-100,000/cm^2 and incubated for 24 hours (12-48 hours) in the above media. Oligonucleotides (20-25mers) are made up in physiological saline and are added to the cultures at 10 μg/ml (10-50 μg/ml = 1.5-7.7 μM).

Protein A sepharose can be obtained from DAKO. Antibodies to Alzheimer's β-amyloid can be obtained from or generated according to U.S. patent serial number 5,231,000, issued July 27, 1993.

Detection of amyloid by solid phase immunoprecipitation

Cells are grown as described except without sera and with 1% bovine serum albumin added. Several concentrations of various oligos including nonsense are used (1-8 μM). Nonsense oligo is used as a control in addition to a control which does not contain any oligos. The controls allow detection of any non-specific response; the nonsense oligo controls additionally permit detection of *per se* toxicity of oligos. ^{35}S-methionine is added at 100 μCi/ml (10-100 μCi/ml). Cells are incubated overnight (6-24 hours).

Beta/A4 is secreted into supernatant of the cell cultures. Hence, the supernatant of the cell cultures are collected and 200 μM cold methionine is added into each to prevent non-specific binding of ^{35}S-methionine. The supernatants are centrifuged at 40,000xg for 30 minutes at room temperature (18-25°C) to remove any cell debris or other extraneous matter. To diminish any non-specific binding of compounds other than amyloid to anti-amyloid antibody which is added in the next step, the supernatant from each centrifuged sample is removed and incubated at room temperature with protein A sepharose (PAS) at 1 mg/ml for 2 hours (1-4 hours) at room temperature. Variations of this procedure would include coupling the anti-amyloid antibody directly to sepharose beads or using a sepharose conjugated secondary such as goat, rabbit, sheep or horse antibodies to whatever species the primary

antibody was made in. However, the protocol would differ slightly for these alternatives. Such adjustments are routine and within the knowledge of those skilled in the art. PAS is removed by centrifugation at 10,000xg for 5 minutes. Antibodies to Alzheimer's β-amyloid (either monoclonal or polyclonal directed to various sequences of β-amyloid amino acids 1-42) are added to the supernatant at 5 μg/ml for monoclonal or similar for polyclonal antibodies, preferably affinity purified polyclonal antibodies. Incubate at room temperature for 2-4 hours. Add Protein A Sepharose as above for 2 hours. The antibody bound PAS is collected and washed several times with phosphate buffered saline containing 0.5% Triton X-100 detergent.

The PAS-amyloid-antibody complex is prepared for separation on a 16% acrylamide gel (Schagger & von Jagow, *Anal. Biochem. 166*:368-379 (1987)). The gel is dried and put against film. The molecular weights of the resulting bands are then determined. Band intensity is measured by densitometry. The level of the 4.3 kD β-amyloid band in the various cell culture samples is compared with the controls; thereby the effect of each antisense oligo on the production of beta-amyloid in the cells as well as the degree of potency of each oligo and the most effective concentration of each oligo in diminishing beta-amyloid production is determined.

Once it is determined which oligos at what concentration are most effective in preventing and/or diminishing the production of amyloid, their therapeutic efficacy can be tested in cell culture as described in Examples 4-7, above. The most effective oligo/NGF combination can then be used for animal and/or human therapy.

Example 9

NGF and/or oligo delivery system. A remote-controlled implantable infusion system is used. A pump that can be refilled transcutaneously and a subcutaneous catheter ending in the lateral ventricle in the brain are used to overcome problems associated with long-term drug delivery to patients with

limited compliance (Harbaugh, R., *Psychopharmacol. Bull. 22*:106-108 (1986); Harbaugh *et al.*, *J. Neurosurg. 71*:481-486 (1989)). The pump system is implanted under general anesthesia using stereotactic surgery and a cylindrical biopsy of cerebral cortex at the parietooccipitotemporal junction is obtained from the site where the NGF- and/or oligo-delivered cannula is to be inserted. The placement of the tip of the cannula is verified by the free flow of cerebrospinal fluid. The programmable pump (Synchromed, Meditronic) is placed in the abdominal wall, and NGF and/or oligo pumping is started immediately. Using a pump rate of 15 μl/h, an NGF dose of approximately 75 μg/24 h is delivered to the patient.

Example 10

NGF preparation. Mouse β-NGF is obtained and tested prior to clinical use as described recently for use in one parkinsonian patient to support adrenal medullary autografts (Olson *et al.*, *Arch. Neurol. 48*:373-381 (1991)). Male adult mouse submandibular glands are used to extract NGF (Chapman *et al.*, *Fed. Eur. Biochem. Soc. Lett. 104*:341-344 (1979); Ebendal *et al.*, in *Cellular and Molecular Biology of Neuronal Development*, I. Black, ed., Plenum, New York (1984), pp. 231-242; Mobley *et al.*, *Biochemistry 15*:5543-5552 (1976)) and the NGF is tested for purity and specific activity. Preferably, the preparation should exert full biological activity (1 BU) *in vitro* in standardized chick embryo ganglia bioassays at a concentration of 5 ng/ml. Prior to clinical use the NGF preparation is sterile-filtered twice, dialyzed against Ringer glucose, and tested for sterility under official standard high-stringency conditions. Parallel batches should also be tested for the presence of pyrogens and foreign genetic material to ascertain that they are free of such contaminants. The NGF preparations should maintain full biological activity for several weeks at 37°C (Olson *et al.*, *Arch. Neurol. 48*:373-381 (1991)).

Example 11

Measurement of NGF and NGF antibodies. Repeated blood samples before, during, and after treatment of the patients are obtained and used to assay for the presence of NGF as well as for the presence of anti-NGF antibodies. These techniques are summarized in a case report (Olson, *J. Neural. Transm. (PD-Sect.) 4*:79-95 (1992); details are given in Lärkfors & Ebendal, *J. Imunol. Methods 97*:31-47 (1987); Olson *et al.*, *Arch. Neurol. 48*:373-381 (1991); Söderström *et al.*, *J. Neurosci. Res. 27*:665-677 (1990)).

Example 12

Cognitive test battery. Seven different tests, monitoring minimental state (Folstein *et al.*, *J. Psychiatr. Res. 12*:189-198 (1975)), face recognition, spatial memory (Sharps & Gollin, *J. Gerontol. 42*:336-341 (1987)), word recognition (Bäckman, L., *Exp. Aging Res. 12*:135-140 (1986)), selective reminding (Buschke, H., *J. Verb. Learn. Verb. Behav. 12*:543-550 (1973)), digit span (Wechsler, D., *Wechsler Adult Intelligence Scale: Manual*, Psychological Corp., New York (1955)), and verbal fluency (Lezak, M., *Neuropsychological Assessment*, Oxford Univ. Press, New York (1983)), respectively, are used to examine any potential effects of treatment on cognitive functioning. (For further description of the test battery and references to these tests, please see Olson, *J. Neural. Transm. (PD-Sect.) 4*:79-95 (1992)).

Example 13

Positron emission tomography. Preparative PET studies include radiolabeled nicotine, radiolabeled butanol, and radiolabeled 2-deoxyglucose. Postoperatively, in order to decrease the amount of radioactivity and stress to

the patient, only nicotine and butanol PET are run. While the blood flow measurements are relatively straightforward, interpretation of nicotine data is more complicated from a modeling point of view, since it is affected not only by specific binding in brain tissue, but also by blood flow and specific and unspecific binding in blood. (For a detailed discussion of these procedures and the interpretation of data, please see Olson *et al.*, *Arch. Neurol. 48*:373-381 (1991) and the work of Nordberg and colleagues (Nordberg *et al.*, *J. Neural. Transm. (PD-Sect.) 2*:215-224 (1990); Nordberg *et al.*, in *Alzheimer's Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies*, K. Iqbal *et al.*, eds., John Wiley, New York (1991), pp. 517-523; Nordberg *et al.*, *J. Neural Transm. (PD-Sect.) 1*:195-205 (1989); Nordberg *et al.*, in *Alzheimer's Disease: Current Research in Early Diagnosis*, Becker & Giacobini, eds., Taylor and Francis, New York (1990), pp. 329-338; Nordberg & Winblad, *Neurosci. Lett. 72*:115-119 (1986)).

Example 14

EEG recordings. Standard techniques with multiple recording sites are employed. EEG recordings are analyzed individually and also using computer-assisted power spectrum analysis.

All publications mentioned above, as well as the references cited in said publications, are herein incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

What Is Claimed Is:

1. A method for reversing morphological changes caused by beta/A4 peptide in a cell, the method comprising the step of administering to the cell an effective amount of an anti-beta/A4 oligonucleotide.

2. A method for treating or preventing Alzheimer's disease in an animal, the method comprising the step of administering to said animal an effective amount of an anti-beta/A4 oligonucleotide.

3. A method for treating down's syndrome in an animal, the method comprising the step of administering to said animal an effective amount of an anti-beta/A4 oligonucleotide.

4. The method according to any one of claims 1-3, wherein the anti-beta/A4 oligonucleotide is complementary to an initiation codon from which beta/A4 peptide is translated.

5. The method according to any one of claims 1-3, wherein the initiation codon is the initiation codon of the APP mRNA.

6. The method according to any one of claims 1-3, wherein the anti-beta/A4 oligonucleotide is complementary to a nucleotide sequence comprising a nucleotide sequence encoding beta/A4 peptide.

7. The method according to claim 6 wherein the nucleotide sequence comprising a nucleotide sequence encoding beta/A4 peptide is the APP gene or RNA.

8. A method for reducing beta/A4 peptide in an animal, including a human, the method comprising the step of administering to the animal an effective amount of an effective amount of an anti-beta/A4 oligonucleotide.

9. The method according to claim 8 wherein the anti-beta/A4 oligonucleotide is complementary to an initiation codon beta/A4 peptide is translated.

10. The method according to claim 9 wherein the initiation codon is the initiation codon of the APP mRNA.

11. The method according to claim 8 wherein the anti-beta/A4 oligonucleotide is complementary to a nucleotide sequence comprising a nucleotide sequence encoding beta/A4 peptide.

12. The method according to claim 11 wherein the nucleotide sequence comprising a nucleotide sequence encoding beta/A4 peptide is the APP gene or RNA.

13. An anti-beta/A4 oligonucleotide that is complementary to an initiation codon from which beta/A4 peptide is translated.

14. The oligonucleotide according to claim 13 wherein the initiation codon is the initiation codon of the APP mRNA.

15. An anti-beta/A4 oligonucleotide that is complementary to a nucleotide sequence comprising a nucleotide sequence encoding beta/A4 peptide.

16. The oligonucleotide according to claim 15 wherein the nucleotide sequence comprising a nucleotide sequence encoding beta/A4 peptide is the APP gene or RNA.

17. The oligonucleotide according to claim 13, wherein the oligonucleotide is a mixed backbone oligonucleotide comprising one or more phosphorothioate or phosphorodithioate regions and one or more alkylphosphonate or alkylphosphonothioate regions.

18. The oligonucleotide according to claim 13, wherein the oligonucleotide is a hybrid oligonucleotide comprising one or more deoxyribonucleotide regions and one or more ribonucleotide regions, and wherein from about one to about all internucleotide linkages are phosphorothioate or phosphorodithioate linkages.

19. The oligonucleotide according to claim 13, wherein the oligonucleotide has at its 3′ end, and optionally at its 5′ end, a cap structure selected from the group consisting of lower alkyl or alcohol groups and the structures shown in Figure 1.

20. The oligonucleotide according to claim 13, wherein the oligonucleotide is a self-stabilized oligonucleotide having a self-complementary region at the 3′ end.

21. The oligonucleotide according to claim 15, wherein the oligonucleotide is a mixed backbone oligonucleotide comprising one or more phosphorothioate or phosphorodithioate region and one or more alkylphosphonate or alkylphosphonothioate region.

22. The oligonucleotide according to claim 15, wherein the oligonucleotide is a hybrid oligonucleotide comprising one or more

deoxyribonucleotide region and one or more ribonucleotide region, and wherein from about one to about all internucleotide linkages are phosphorothioate or phosphorodithioate linkages.

23. The oligonucleotide according to claim 15, wherein the oligonucleotide has at its 3′ end, and optionally at its 5′ end, a cap structure selected from the group consisting of lower alkyl or alcohol groups and the structures shown in Figure 1.

24. The oligonucleotide according to claim 15, wherein the oligonucleotide is a self-stabilized oligonucleotide having a self-complementary region at the 3′ end.

25. A pharmaceutical composition comprising the oligonucleotide of claim 13 or 15 and a pharmaceutically acceptable carrier.

26. A composition of matter which comprises nerve growth factor and an anti-beta/A4 oligonucleotide.

27. The composition of matter as claimed in claim 26, wherein the anti-beta/A4 oligonucleotide is complementary to an initiation codon from which beta/A4 peptide is translated.

28. The composition of matter as claimed in claim 27, wherein the initiation codon is the initiation codon of APP mRNA.

29. The composition of matter as claimed in claim 26, wherein the anti-beta/A4 oligonucleotide is complementary to a nucleotide sequence which comprises a nucleotide sequence encoding beta/A4 peptide.

30. The composition of matter as claimed in claim 29, wherein the nucleotide sequence which comprises a nucleotide sequence encoding beta/A4 peptide is APP gene or RNA.

31. The composition of matter as claimed in claim 27, wherein said oligonucleotide is a mixed backbone oligonucleotide comprising one or more phosphorothioate or phosphorothioate regions and one or more alkylphosphonate or alkylphosphonothioate regions.

32. The composition of matter as claimed in claim 27, wherein said oligonucleotide is a hybrid oligonucleotide which comprises one or more deoxyribonucleotide regions and one or more ribonucleotide regions, and wherein from about one to about all internucleotide linkages are phosphorothioate or phosphorodithioate linkages.

33. The composition of matter as claimed in claim 27, wherein said oligonucleotide has at its 3' end, a cap structure selected from the group consisting of lower alkyl or alcohol groups and the structure shown in Figure 1.

34. The composition of matter as claimed in claim 27, wherein said oligonucleotide has at its 3' end and at its 5' end, a cap structure selected from the group consisting of lower alkyl or alcohol groups and the structure shown in Figure 1.

35. The composition of matter as claimed in claim 27, wherein said oligonucleotide is a self-stabilized oligonucleotide having a self-complementary region at its 3' end.

36. The composition of matter as claimed in claim 29, wherein said oligonucleotide is a mixed backbone oligonucleotide comprising one or more

phosphorothioate or phosphorothioate regions and one or more alkylphosphonate or alkylphosphonothioate regions.

37. The composition of matter as claimed in claim 29, wherein said oligonucleotide is a hybrid oligonucleotide which comprises one or more deoxyribonucleotide regions and one or more ribonucleotide regions, and wherein from about one to about all internucleotide linkages are phosphorothioate or phosphorodithioate linkages.

38. The composition of matter as claimed in claim 29, wherein said oligonucleotide has at its 3' end, a cap structure selected from the group consisting of lower alkyl or alcohol groups and the structure shown in Figure 1.

39. The composition of matter as claimed in claim 29, wherein said oligonucleotide has at its 3' end and at its 5' end, a cap structure selected from the group consisting of lower alkyl or alcohol groups and the structure shown in Figure 1.

40. The composition of matter as claimed in claim 29, wherein said oligonucleotide is a self-stabilized oligonucleotide having a self-complementary region at its 3' end.

41. A pharmaceutical composition which comprises a composition of matter as claimed in any of the claims 26-40 in a pharmaceutically acceptable carrier.

42. A kit for a pharmaceutical administration to patients in need thereof which comprises a carrier means being compartmentalized to receive in close confinement therein one or more container means wherein

(a) the first container means contains a beta/A4 antisense oligonucleotide; and

(b) a second container means contains a growth factor.

43. The kit of claim 42, wherein said antisense oligonucleotides are present as a solution in an aqueous buffer or a physiological solution.

44. The kit of claim 42, wherein said growth factor is nerve growth factor.

45. The kit of claim 42, wherein said growth factor is epidermal growth factor.

46. The kit of claim 44, wherein said nerve growth factor is present as a solution in an aqueous buffer or a physiological solution.

47. The kit of claim 44, wherein said nerve growth factor is present in lyophilized form.

48. The kit of claim 42, further comprising

(c) one or more container means containing a different growth factor than that used in step (b), present as a solution in an aqueous buffer or a physiological solution.

49. The kit of claim 42, further comprising

(c) one or more container means containing a different growth factor than that used in step (b), present as a solution in an aqueous buffer or a physiological solution.

50. A method for treatment of amyloidosis which comprises administering an effective amount of a pharmaceutical composition as claimed in claim 41 to a patient in need thereof.

51. A method for treatment of amyloidosis in a patient which comprises

(a) administering an effective amount of an beta/A4 amyloid antisense oligo to a patient in need thereof; and

(b) increasing levels of NGF in parts or all of the central nervous system of the patient to stimulate neurotrophic effects in said patient.

52. A method as claimed in claim 51, wherein the levels of NGF are increased by grafting NGF-producing cells to intracerebral sites.

53. A method as claimed in claim 51, wherein the levels of NGF are increased by transferring the genes necessary to produce NGF into nondividing neuronal or gland cells.

54. A method as claimed in claim 51, wherein the levels of NGF are increased by intracerebral NGF infusion.

55. A method as claimed in claim 51, wherein the levels of NGF are increased by implantation of a slow-release biodegradable implant.

56. A method as claimed in claim 51, wherein the levels of NGF are increased by carrier-mediated transport across blood-brain barrier.

57. An assay for screening candidate antisense oligonucleotides effective in treatment of deleterious effects that are visited upon cells by beta/A4 amyloid peptide, which comprises

(a) plating several containers of mammalian neuronal cell cultures;

(b) making test samples by adding various concentrations of different beta/A4 antisense oligos to several of the containers plated in step (a);

(c) making control samples by adding either no oligos or non-sense oligos to several other containers plated in step (a);

(d) incubating the test samples and the controls with labelled methionine about 6 to about 24 hours;

(e) collecting supernatant from each container;

(f) contacting the supernatant from each container with protein A sepharose (PAS) to form a PAS-amyloid complex;

(g) contacting the PAS-amyloid complex with an antibody to beta-amyloid to form an PAS-amyloid-antibody complex;

(h) separating the PAS-amyloid-antibody on an acrylamide gel by electrophoresis to form bands of PAS-amyloid-antibody complex corresponding to each cell culture;

(i) determining levels of 4.3 KD beta-amyloid present in the bands by densitometry;

(j) comparing the levels of 4.3 KD beta-amyloid present in test samples with the control samples thereby determining effectiveness of the beta/A4 antisense oligos in reducing production of amyloid in said cells;

(k) selecting those oligonucleotides from step (j) which are most effective in reducing amyloid production;

(l) determining effect of addition of said oligonucleotides, which were selected in step (j), to mammalian neuronal cells treated with a growth factor, which stimulates growth and differentiation of said neuronal cells; and

(m) selecting those oligonucleotides from step (l) which do not inhibit trophic effect of said growth factor.

58. An assay as claimed in claim 57, wherein said growth factor is neuronal growth factor.

59. An assay as claimed in claim 58, wherein said neuronal growth factor is a human neuronal growth factor.

60. An assay as claimed in claim 58, wherein said neuronal growth factor is a murine neuronal growth factor.

61. An assay as claimed in claim 57, wherein said growth factor is epidermal growth factor.

62. An assay as claimed in claim 61, wherein said epidermal growth factor is a human epidermal growth factor.

63. An assay as claimed in claim 61, wherein said epidermal growth factor is a murine epidermal growth factor.

64. An assay as claimed in claim 57, wherein said mammalian neuronal cell line is PC12.

65. An assay as claimed in claim 57, wherein said mammalian neuronal cell line is IMR-32.

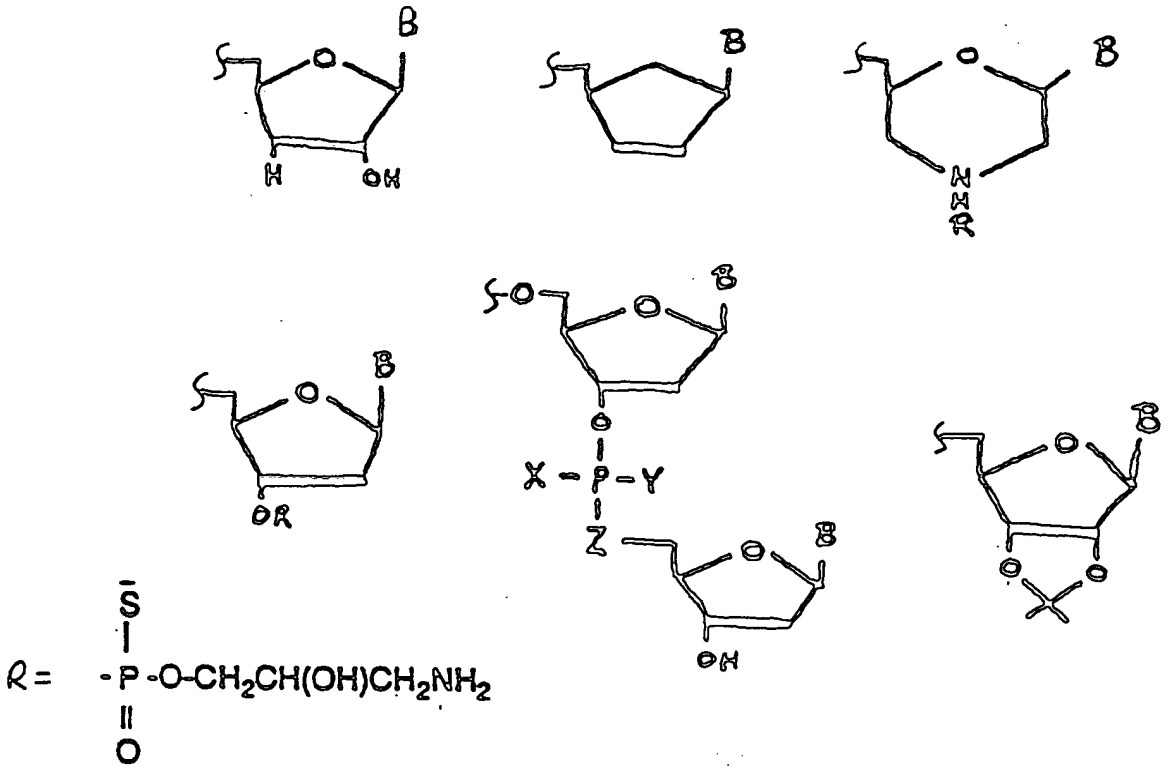


Figure 1

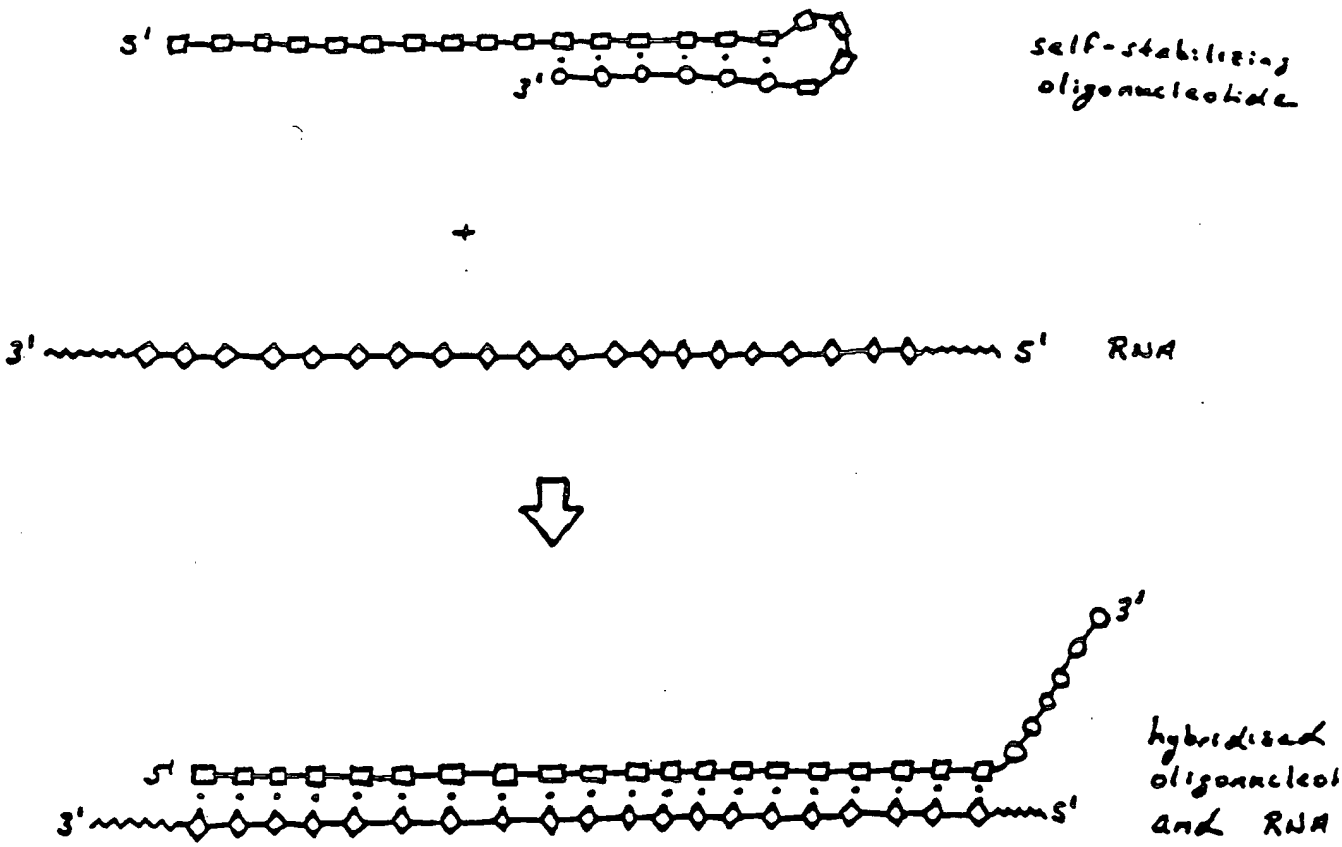

5'
3'
self-stabilizing oligonucleotide
+
3'
5'
RNA
3'
5'
5'
3'
hybridized oligonucleotide and RNA

Figure 2

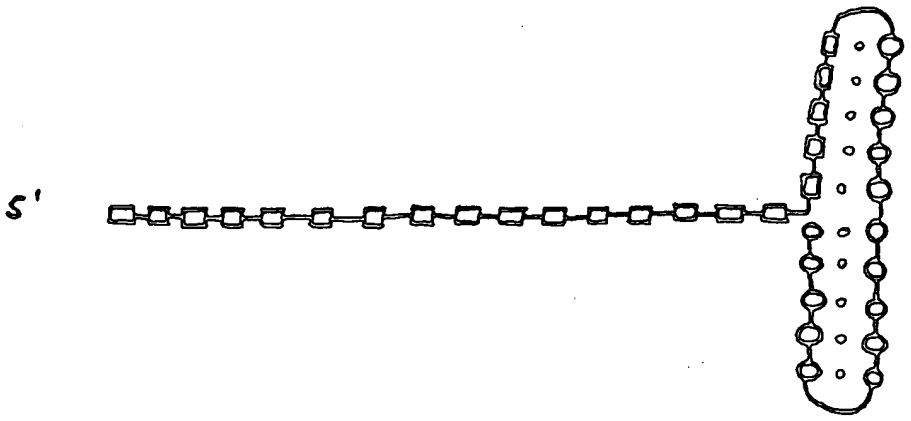


Figure 3

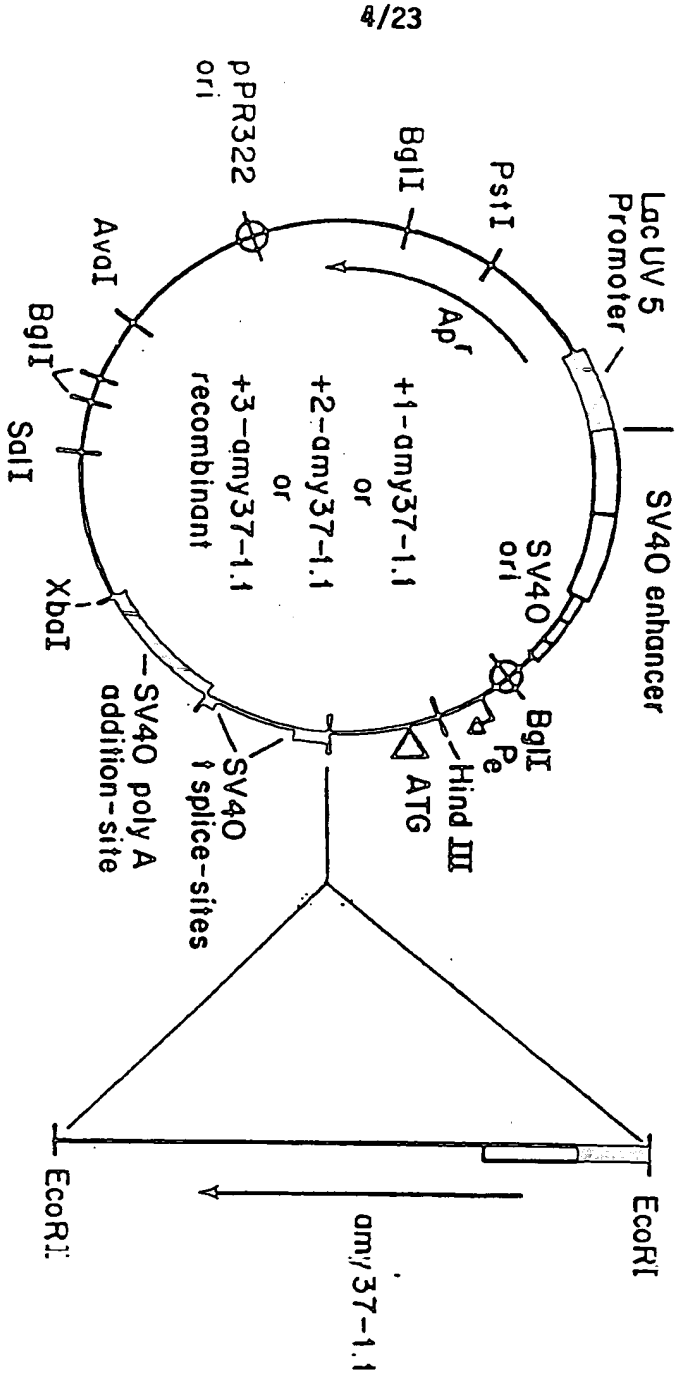

pPR322 ori
BglI
PstI
LacUV 5 Promoter
XbaI
SV40 enhancer
AvaI
BglI
SalI
XbaI
Ap^r
+1-amy37-1.1
or
+2-amy37-1.1
or
+3-amy37-1.1
recombinant
SV40 ori
BglII
Pe
Hind III
ATG
SV40 poly A addition-site
SV40 splice-sites
EcoRI
EcoRI
amy37-1.1

Figure 4

Figure 5A

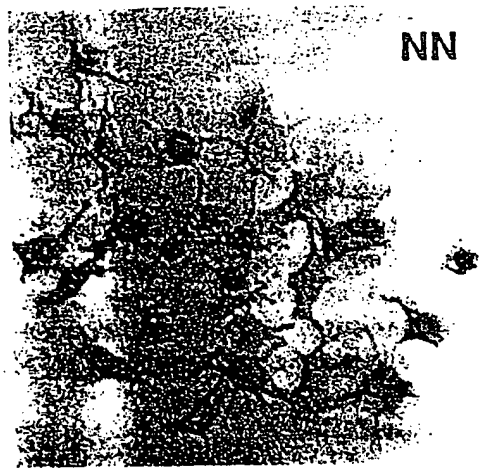


Figure 5B

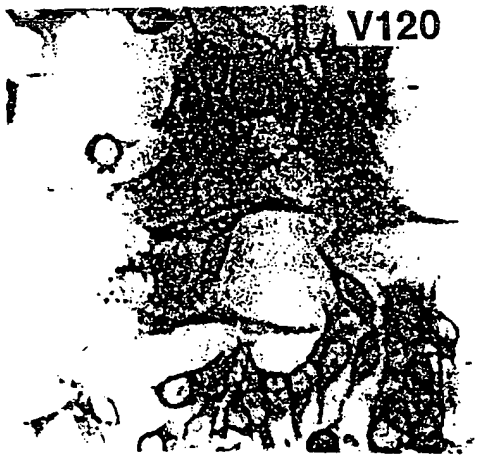


Figure 5C

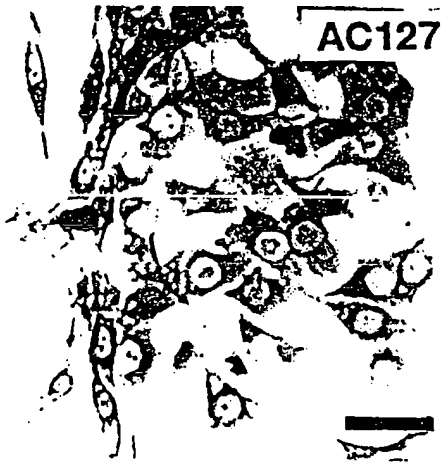


Figure 6A

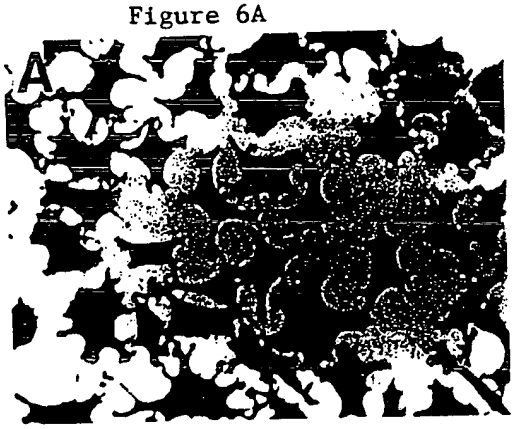


Figure 6B

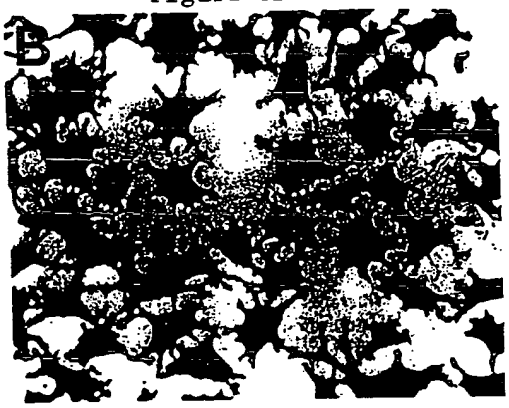




Figure 6C



Figure 6D

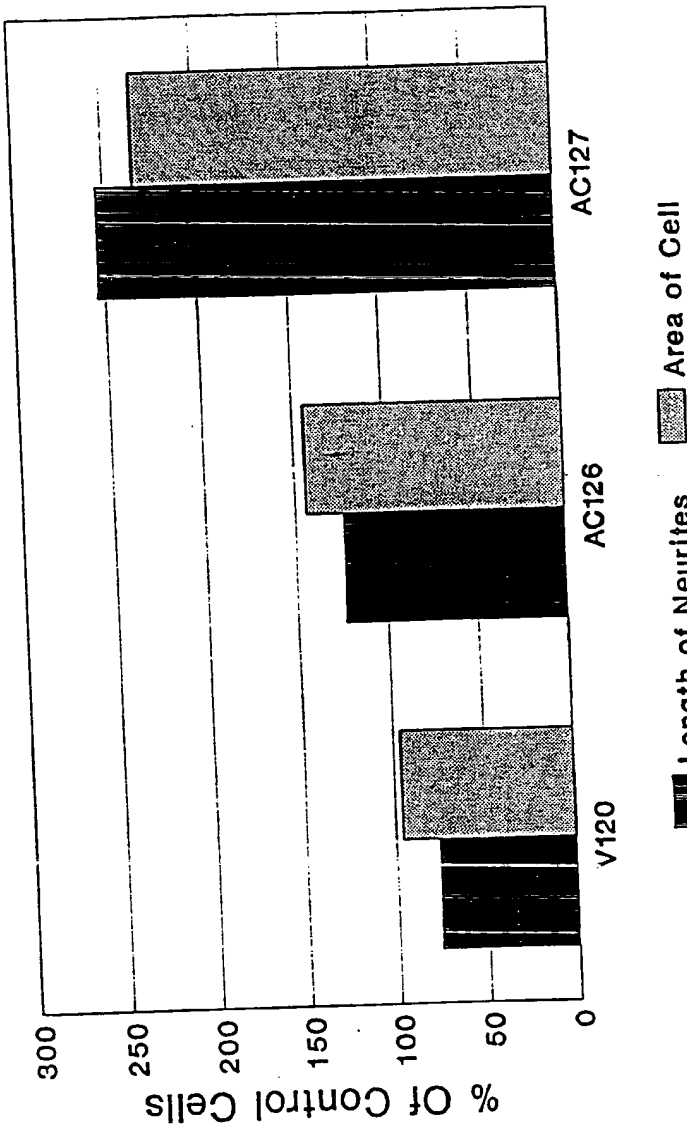
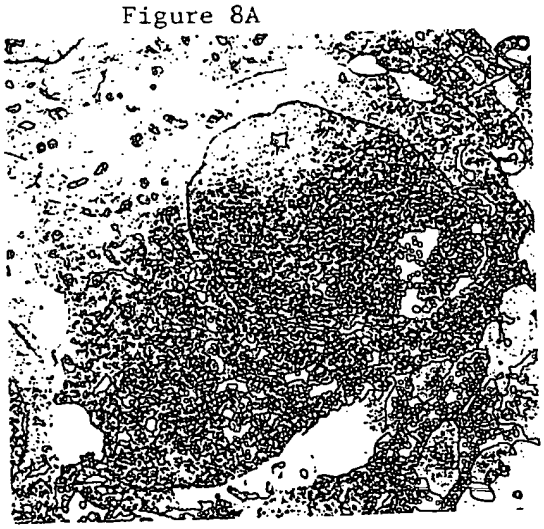

% Of Control Cells
300
250
200
150
100
50
0
V120
AC126
AC127
Length of Neurites
Area of Cell

Figure 7

Figure 8A



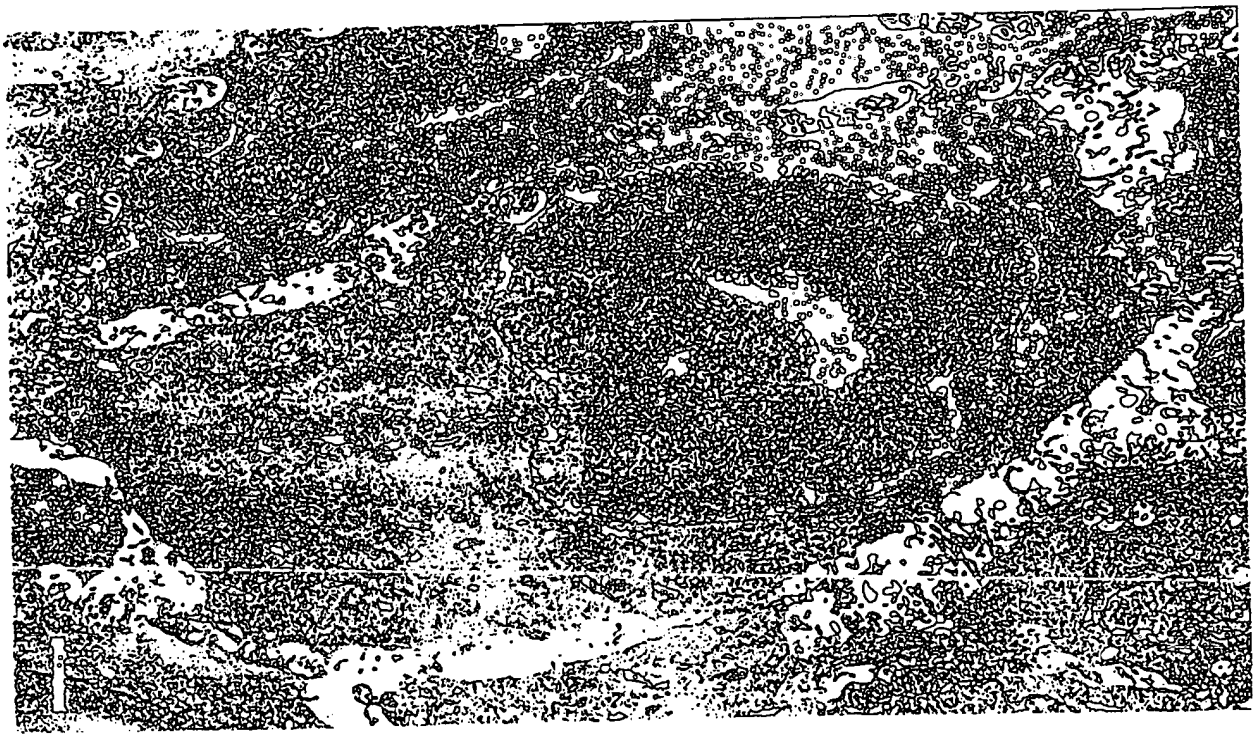


Figure 8B

Figure 9A

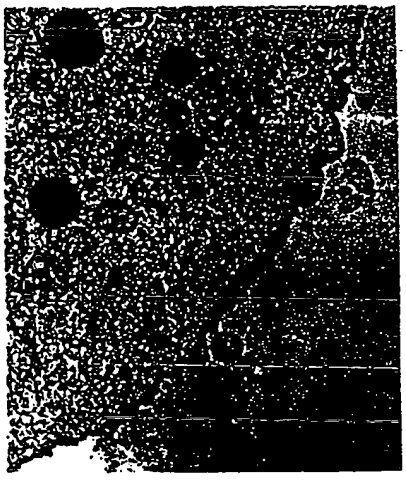


Figure 9B





Figure 9C



Figure 9D

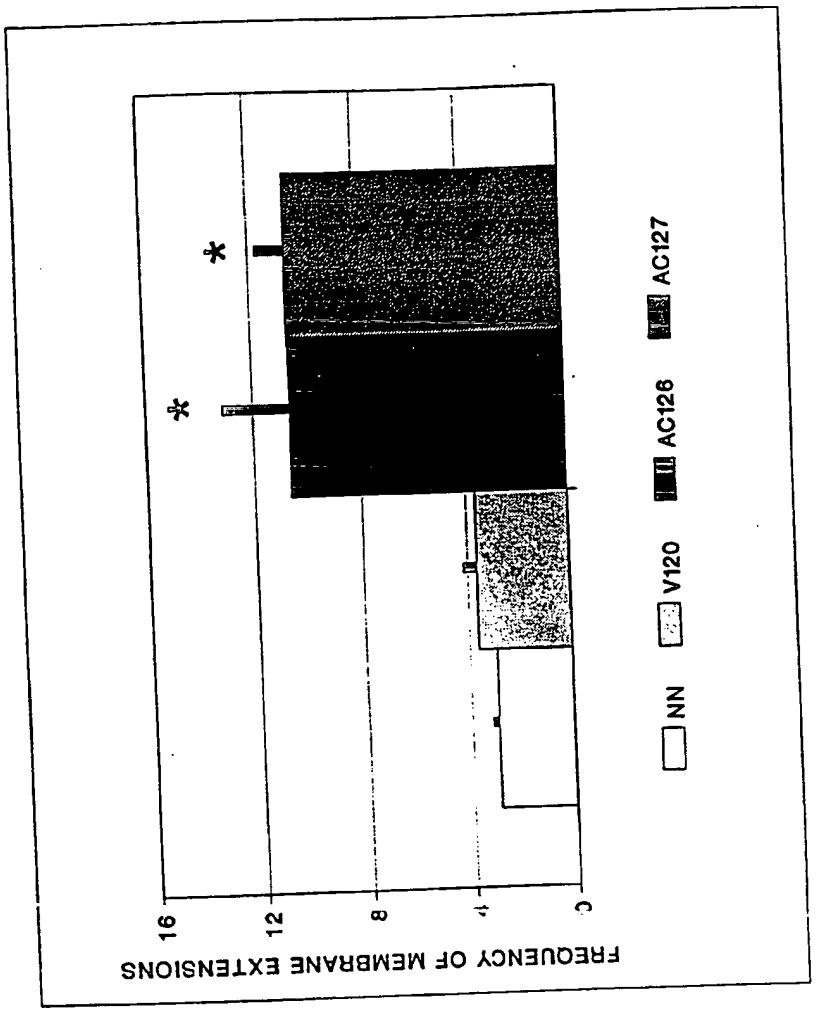

FREQUENCY OF MEMBRANE EXTENSIONS
16
12
8
4
0
*
*
NN
V120
AC126
AC127

Figure 10

Figure 11A

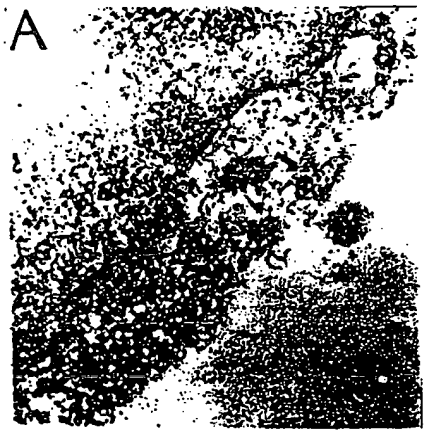


Figure 11B



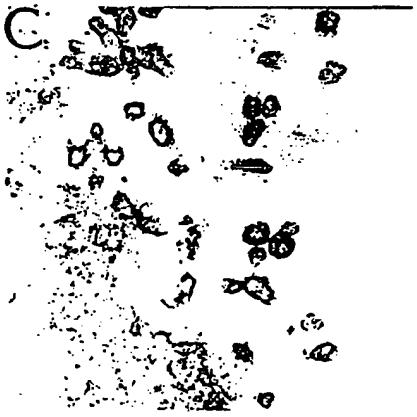


Figure 11C

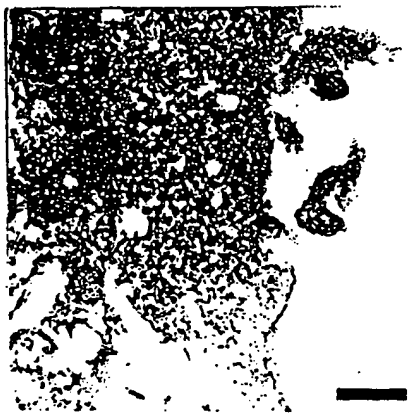


Figure 11D



Figure 12A



Figure 12B

AGTTTCCTCG GCAGCGGTAG GCGAGAGCAC GCGGAGGAGC GTGCGCGGGG CCCCGGGAGA 60

CGGCGGCGGT GGCGGCGCGG GCAGAGCAAG GACGCGGCGG ATCCCACTCG CACAGCAGCG 120

CACTCGGTGC CCCGCGCAGG GTCGCGATGC TGCCCGGTTT GGCACTGCTC CTGCTGGCCG 180

CCTGGACGGC TCGGGCGCTG GAGGTACCCA CTGATGGTAA TGCTGGCCTG CTGGCTGAAC 240

CCCAGATTGC CATGTTCTGT GGCAGACTGA ACATGCACAT GAATGTCCAG AATGGGAAGT 300

GGGATTCAGA TCCATCAGGG ACCAAAACCT GCATTGATAC CAAGGAAGGC ATCCTGCAGT 360

ATTGCCAAGA AGTCTACCCT GAACTGCAGA TCACCAATGT GGTAGAAGCC AACCAACCAG 420

TGACCATCCA GAACTGGTGC AAGCGGGGCC GCAAGCAGTG CAAGACCCAT CCCCACTTTG 480

TGATTCCCTA CCGCTGCTTA GTTGGTGAGT TTGTAAGTGA TGCCCTTCTC GTTCCTGACA 540

AGTGCAAATT CTTACACCAG GAGAGGATGG ATGTTTGCGA AACTCATCTT CACTGGCACA 600

CCGTCGCCAA AGAGACATGC AGTGAGAAGA GTACCAACTT GCATGACTAC GGCATGTTGC 660

TGCCCTGCGG AATTGACAAG TTCCGAGGGG TAGAGTTTGT GTGTTGCCCA CTGGCTGAAG 720

AAAGTGACAA TGTGGATTCT GCTGATGCGG AGGAGGATGA CTCGGATGTC TGGTGGGGCG 780

GAGCAGACAC AGACTATGCA GATGGGAGTG AAGACAAAGT AGTAGAAGTA GCAGAGGAGG 840

AAGAAGTGGC TGAGGTGGAA GAAGAAGAAG CCGATGATGA CGAGGACGAT GAGGATGGTG 900

ATGAGGTAGA GGAAGAGGCT GAGGAACCCT ACGAAGAAGC CACAGAGAGA ACCACCAGCA 960

TTGCCACCAC CACCACCACC ACCACAGAGT CTGTGGAAGA GGTGGTTCGA GTTCCTACAA 1020

CAGCAGCCAG TACCCCTGAT GCCGTTGACA AGTATCTCGA GACACCTGGG GATGAGAATG 1080

AACATGCCCA TTTCCAGAAA GCCAAAGAGA GGCTTGAGGC CAAGCACCGA GAGAGAATGT 1140

CCCAGGTCAT GAGAGAATGG GAAGAGGCAG AACGTCAAGC AAAGAACTTG CCTAAAGCTG 1200

ATAAGAAGGC AGTTATCCAG CATTTCCAGG AGAAAGTGGA ATCTTTGGAA CAGGAAGCAG 1260

CCAACGAGAG ACAGCAGCTG GTGGAGACAC ACATGGCCAG AGTGGAAGCC ATGCTCAATG 1320

ACCGCCGCCG CCTGGCCCTG GAGAACTACA TCACCGCTCT GCAGGCTGTT CCTCCTCGGC 1380

CTCGTCACGT GTTCAATATG CTAAAGAAGT ATGTCCGCGC AGAACAGAAG GACAGACAGC 1440

ACACCCTAAA GCATTTCGAG CATGTGCGCA TGGTGGATCC CAAGAAAGCC GCTCAGATCC 1500

GGTCCCAGGT TATGACACAC CTCCGTGTGA TTTATGAGCG CATGAATCAG TCTCTCTCCC 1560

Figure 13A

TGCTCTACAA CGTGCCTGCA GTGGCCGAGG AGATTCAGGA TGAAGTTGAT GAGCTGCTTC 1620

AGAAAGAGCA AAACTATTCA GATGACGTCT TGGCCAACAT GATTAGTGAA CCAAGGATCA 1680

GTTACGGAAA CGATGCTCTC ATGCCATCTT TGACCGAAAC GAAAACCACC GTGGAGCTCC 1740

TTCCCGTGAA TGGAGAGTTC AGCCTGGACG ATCTCCAGCC GTGGCATTCT TTTGGGGCTG 1800

ACTCTGTGCC AGCCAACACA GAAAACGAAG TTGAGCCTGT TGATGCCCGC CCTGCTGCCG 1860

ACCGAGGACT GACCACTCGA CCAGGTTCTG GGTTGACAAA TATCAAGACG GAGGAGATCT 1920

CTGAAGTGAA GATGGATGCA GAATTCCGAC ATGACTCAGG ATATGAAGTT CATCATCAAA 1980

AATTGGTGTT CTTTGCAGAA GATGTGGGTT CAAACAAAGG TGCAATCATT GGACTCATGG 2040

TGGGCGGTGT TGTCATAGCG ACAGTGATCG TCATCACCTT GGTGATGCTG AAGAAGAAAC 2100

AGTACACATC CATTCATCAT GGTGTGGTGG AGGTTGACGC CGCTGTCACC CCAGAGGAGC 2160

GCCACCTGTC CAAGATGCAG CAGAACGGCT ACGAAAATCC AACCTACAAG TTCTTTGAGC 2220

AGATGCAGAA CTAGACCCCC GCCACAGCAG CCTCTGAAGT TGGACAGCAA AACCATTGCT 2280

TCACTACCCA TCGGTGTCCA TTTATAGAAT AATGTGGGAA GAAACAAACC CGTTTTATGA 2340

TTTACTCATT ATCGCCTTTT GACAGCTGTG CTGTAACACA AGTAGATGCC TGAACTTGAA 2400

TTAATCCACA CATCAGTAAT GTATTCTATC TCTCTTTACA TTTTGGTCTC TATACTACAT 2460

TATTAATGGG TTTTGTGTAC TGTAAAGAAT TTAGCTGTAT CAAACTAGTG CATGAATAGA 2520

TTCTCTCCTG ATTATTTATC ACATAGCCCC TTAGCCAGTT GTATATTATT CTTGTGGTTT 2580

GTGACCCAAT TAAGTCCTAC TTTACATATG CTTTAAGAAT CGATGGGGGA TGCTTCATGT 2640

GAACGTGGGA GTTCAGCTGC TTCTCTTGCC TAAGTATTCC TTTCCTGATC ACTATGCATT 2700

TTAAAGTTAA ACATTTTTAA GTATTTCAGA TGCTTTAGAG AGATTTTTTT TCCATGACTG 2760

CATTTTACTG TACAGATTGC TGCTTCTGCT ATATTTGTGA TATAGGAATT AAGAGGATAC 2820

ACACGTTTGT TTCTTCGTGC CTGTTTTATG TGCACACATT AGGCATTGAG ACTTCAAGCT 2880

TTTCTTTTTT TGTCCACGTA TCTTTGGGTC TTTGATAAAG AAAAGAATCC CTGTTCATTG 2940

TAAGCACTTT TACGGGGCGG GTGGGGAGGG GTGCTCTGCT GGTCTTCAAT TACCAAGAAT 3000

TCTCCAAAAC AATTTTCTGC AGGATGATTG TACAGAATCA TTGCTTATGA CATGATCGCT 3060

TTCTACACTG TATTACATAA ATAAATTAAA TAAAATAACC CCGGGCAAGA CTTTTCTTTG 3120

AAGGATGACT ACAGACATTA AATAATCGAA GTAATTTTGG GTGGGGAGAA GAGGCAGATT 3180

CAATTTTCTT TAACCAGTCT GAAGTTTCAT TTATGATACA AAAGAAGATG AAAATGGAAG 3240

TGGCAATATA AGGGGATGAG GAAGGCATGC CTGGACAAAC CCTTCTTTTA AGATGTGTCT 3300

TCAATTTGTA TAAAATGGTG TTTTCATGTA AATAAATACA TTCTTGGAGG AGC 3353

Figure 13B



Figure 14A



Figure 14B

Figure 15A

Figure 15B

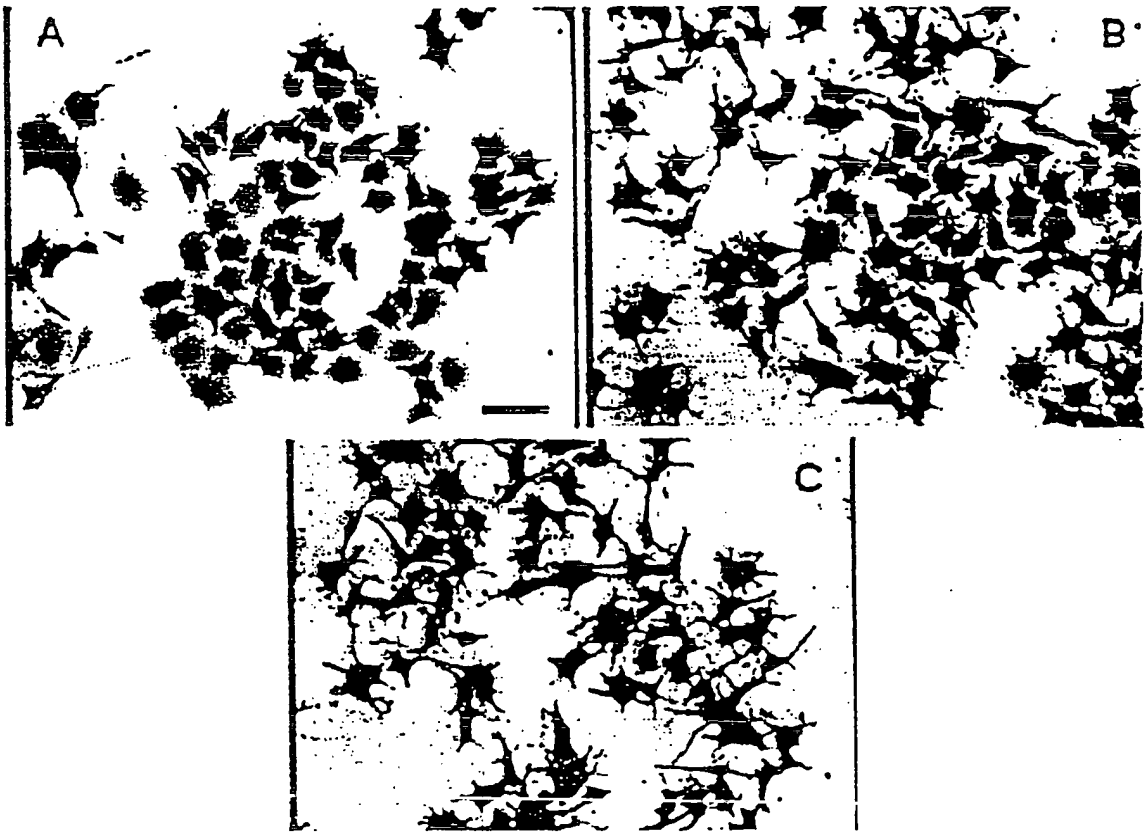


Figure 15C

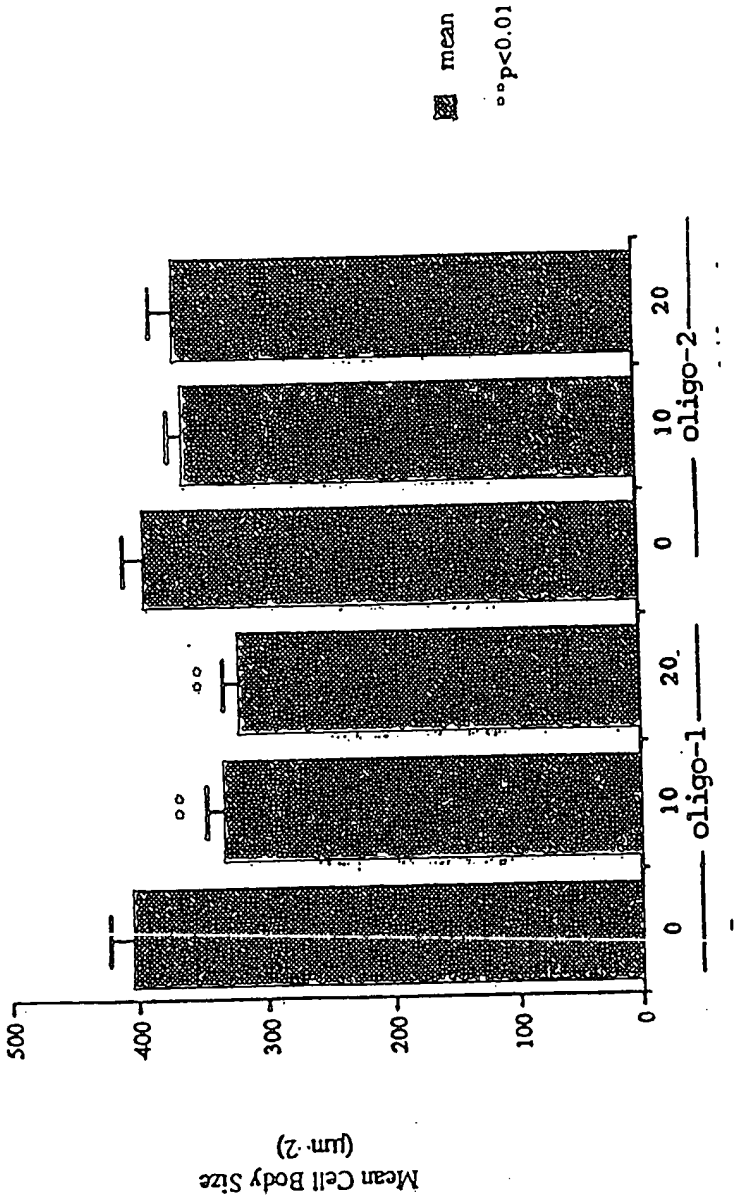

mean
°°p<0.01
Mean Cell Body Size
(μm·2)
500
400
300
200
100
0
°°
°°
0
10
20
oligo-1
0
10
20
oligo-2

Figure 16

Figure 17A

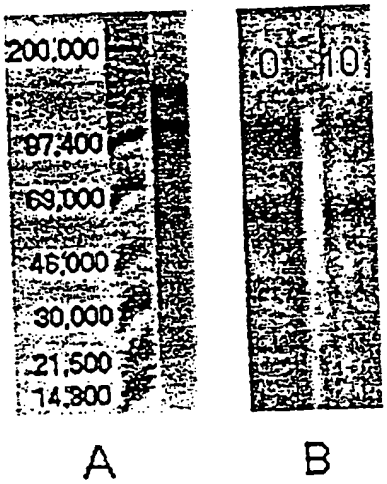


Figure 17C

Figure 17B

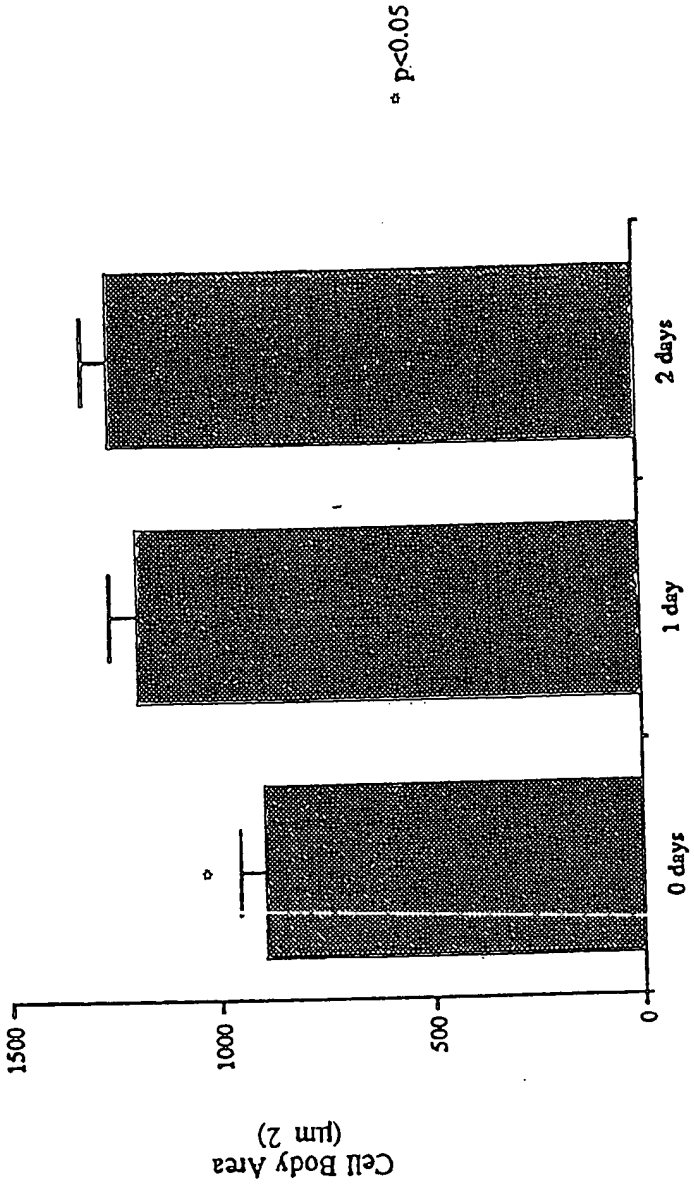


Figure 18

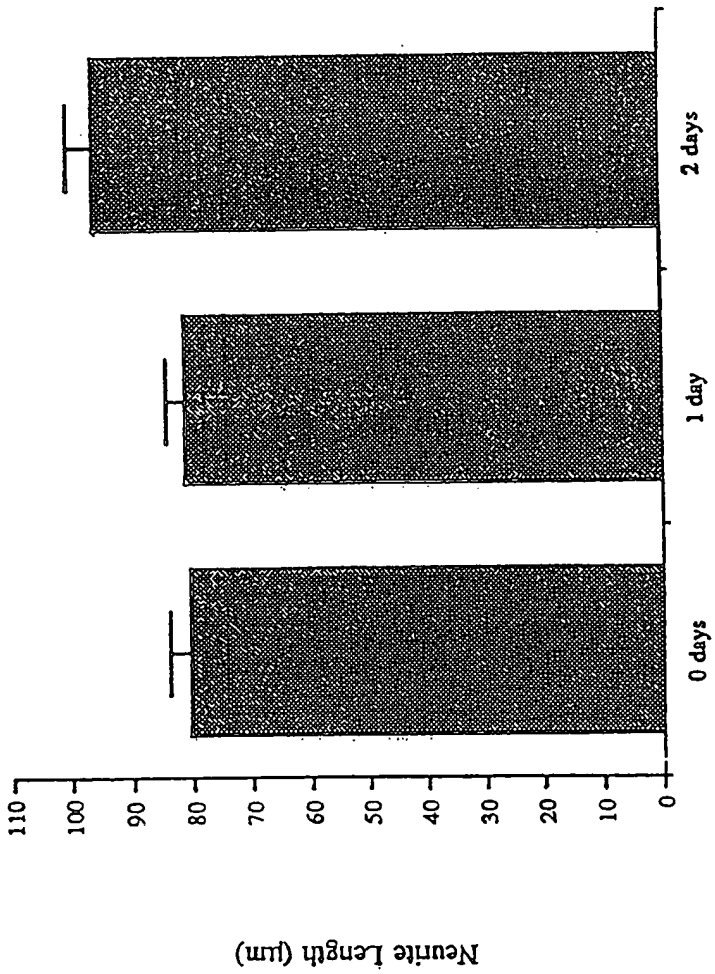

Neurite Length (μm)
110
100
90
80
70
60
50
40
30
20
10
0
0 days
1 day
2 days

Figure 19

** p<0.01
Cell Area (μm)
1500
1000
500
0
0
5
10
15
oligo-1
0
15
oligo-2
μg/ml
**

Figure 20

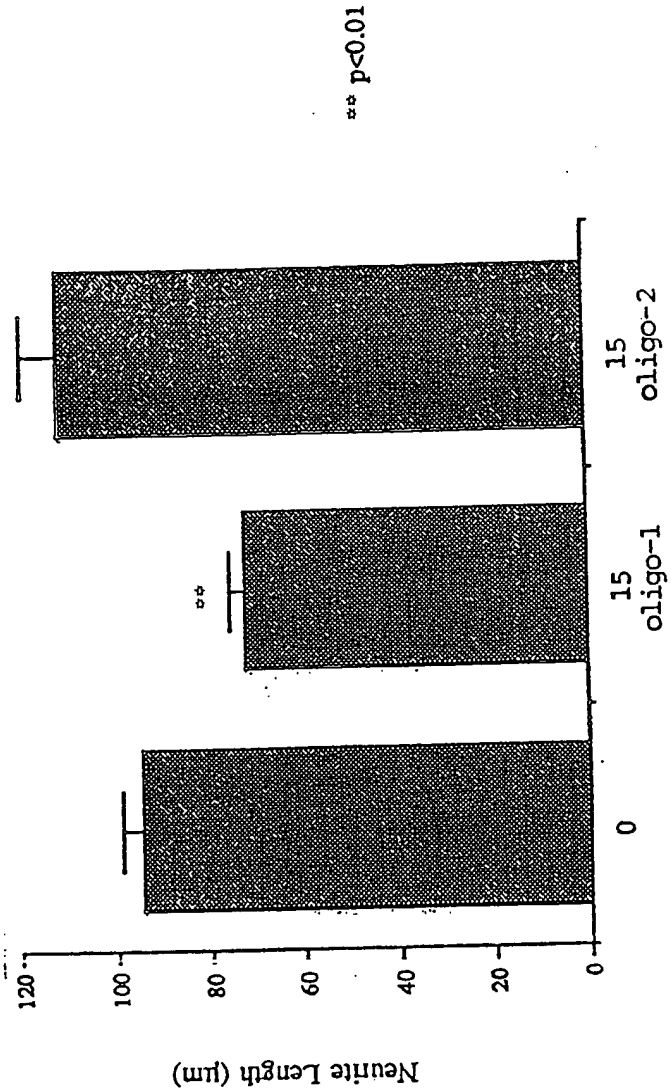

** p<0.01
Neurite Length (μm)
120
100
80
60
40
20
0
**
0
15
oligo-1
15
oligo-2
pg/ml

Figure 21

Figure 22A

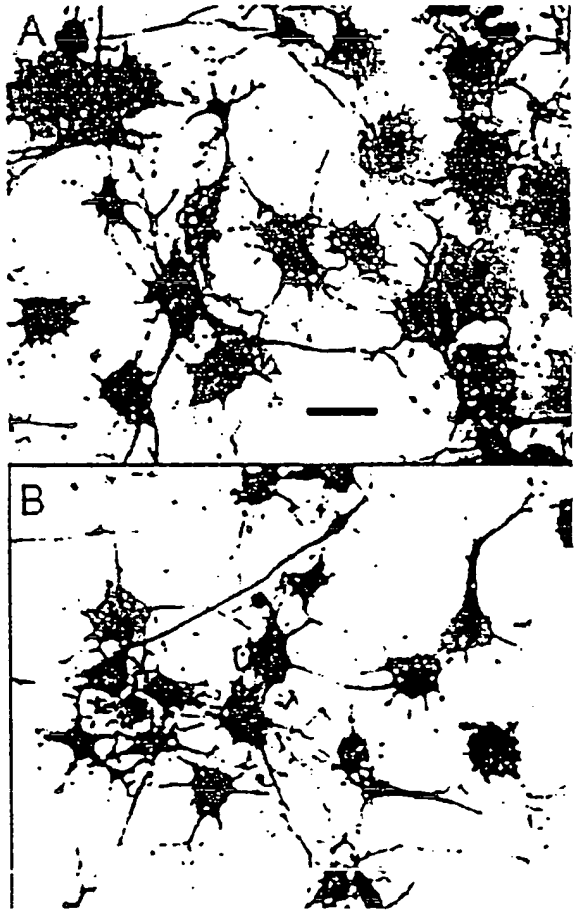


Figure 22B

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 94/10943

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 A61K31/70 C07H21/00 A61K38/18 C12N5/10
C12Q1/68 A61K9/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 13114 (ISIS PHARMACEUTICALS, INC.) 8 July 1993 see page 3, line 9 - page 5, line 2	1-16,25
Y	see page 7, line 21 - page 9, line 30 see examples 6-7 see claims ---	17-24, 26-65
Y	WO,A,91 12323 (WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY) 22 August 1991 see page 2, line 27 - page 3, line 14 see page 6, line 1 - line 18 see table 2 see claims 1,4,5,10,12 --- -/--	17,18, 21,22, 31,32, 36,37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 1995	16.3.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, vol.19, no.4, 1991, ARLINGTON, VIRGINIA US pages 747 - 750 SHAW, J-P ET AL. 'Modified deoxyoligonucleotides stable to exonuclease degradation in serum' see figure 1 ---	19,23, 33,34, 38,39
Y	NUCLEIC ACIDS RESEARCH, vol.21, 11 June 1993, ARLINGTON, VIRGINIA US pages 2729 - 2735 TANG, J. ET AL. 'Self-stabilized antisense oligodeoxynucleotide phosphorothioates: properties and anti-HIV activity' see the whole document ---	20,24, 35,40
X	J.NEUROSIENCE RESEARCH, vol.31, no.4, April 1992 pages 635 - 645 LEBLANC, A. ET AL. 'Role of amyloid precursor protein (APP): study with antisense transfection of human neuroblastoma cells' ---	15
X	WO,A,93 03743 (THE GENERAL HOSPITAL CORPORATION ET AL.) 4 March 1993	1,2
A	see page 10, line 17 - page 12, line 14 ---	26-56
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol.164, no.2, 31 October 1989, DULUTH, MINNESOTA US pages 664 - 670 REFOLO, L. ET AL. 'Nerve and epidermal growth factors induce the release of the Alzheimer amyloid precursor from PC12 cell cultures' cited in the application see the whole document ---	26-56
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.86, January 1989, WASHINGTON US pages 337 - 341 MAROTTA, C. ET AL. 'Overexpression of amyloid precursor protein A4 (beta-amyloid) immunoreactivity in genetically transformed cells: implications for a cellular model of Alzheimer amyloidosis' cited in the application see the whole document ---	57-65

-/--

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC NATL ACAD SCI U S A 86 (12). 1989. 4756-4760 ERNFORS, P. ET AL. 'A CELL LINE PRODUCING RECOMBINANT NERVE GROWTH FACTOR EVOKES GROWTH RESPONSES IN INTRINSIC AND GRAFTED CENTRAL CHOLINERGIC NEURONS.' cited in the application see the whole document	52,53
A	SCIENCE (WASHINGTON D C) 259 (5093). 373-377, 15 January 1993 FRIDEN, P. ET AL. 'BLOOD - BRAIN BARRIER PENETRATION AND IN-VIVO ACTIVITY OF AN NGF CONJUGATE.' cited in the application see the whole document	56
A	NEUROSURGERY (BALTIMORE) 30 (3). 1992. 313-319 CAMARATA, P. ET AL. 'SUSTAINED RELEASE OF NERVE GROWTH FACTOR FROM BIODEGRADABLE POLYMER MICROSPHERES.' cited in the application see the whole document	55
A	NEURON, vol.9, no.1, July 1992 pages 129 - 137 MILWARD, E. ET AL. 'The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth' cited in the application	
T	EXPERIMENTAL NEUROLOGY 124 (1). 5-15, November 1993 OLSON, L. 'NGF and the treatment of Alzheimer 's disease.' see the whole document	26-56

INTERNATIONAL SEARCH REPORT

Ir. .ational application No.
PCT/US 94/ 10943

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-12 and 50-57 (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[] The additional search fees were accompanied by the applicant's protest.

[] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 94/10943

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9313114	08-07-93	AU-B-	3249793	28-07-93
		CA-A-	2126451	08-07-93
		JP-T-	6511387	22-12-94
WO-A-9112323	22-08-91	US-A-	5149797	22-09-92
		EP-A-	0515511	02-12-92
		US-A-	5366878	22-11-94
		US-A-	5220007	15-06-93
WO-A-9303743	04-03-93	AU-A-	2468992	16-03-93